Is flaxseed equivalent and/or synergistic with ACE inhibition in the prevention of chemotherapy induced cardiotoxicity?

by

Cameron Robert Eekhoudt

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Department of Physiology and Pathophysiology

Max Rady College of Medicine, Rady Faculty of Health Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

Abstract

Background: While Doxorubicin (DOX) and Trastuzumab (TRZ) are two of the most common anti-neoplastic agents used in the treatment of breast cancer, there are cardiotoxic side effects associated with their use. Recent studies have evaluated the role of pharmaceutical agents, such as perindopril (PER), and nutraceutical agents, such as flaxseed (FLX), in the prevention of DOX+TRZ mediated cardiotoxicity. Little is known, however, on whether FLX will work comparably and/or synergistically with PER in preventing chemotherapy-induced cardiotoxicity.

Objective: To determine whether prophylactic administration of FLX will work equivalently and/or synergistically with PER in the prevention of DOX+TRZ-mediated cardiotoxicity in a chronic *in vivo* female murine model.

Methods: A total of 200 wild-type C57Bl/6 female mice were randomized to receive either regular chow (RC) or FLX-supplemented diets for a total of 6 weeks. On weeks 4, 5, and 6, mice were further randomized to receive an intraperitoneal injection of: i) 0.9% saline; ii) DOX (8mg/kg/wk); iii) TRZ (3mg/kg/wk); or iv) DOX+TRZ to create a chronic *in vivo* murine model of chemotherapy-induced cardiotoxicity. Within each group, mice were randomized to receive PER (3mg/kg/daily) daily via oral gavage. Weekly echocardiography and hemodynamic parameters were measured throughout the 6-week study. At study endpoint, cardiac tissues were harvested for histological and biochemical analyses.

Results and Discussion: In mice treated with RC+DOX+TRZ, left ventricular end diastolic diameter (LVEDD) increased from 2.8±0.2 mm at baseline to 4.3±0.2 mm by week 6. Prophylactic administration of either PER or FLX alone partially prevented adverse left ventricular (LV)

remodelling with LVEDD values of 3.4±0.3 mm and 3.5±0.2 mm, respectively. Similarly, the left ventricular ejection fraction (LVEF) in mice treated with RC+DOX+TRZ decreased from 75±2% at baseline to 37±3% at week 6. Prophylactic treatment with either PER or FLX alone partially attenuated LV systolic dysfunction with LVEF values of 63±2% and 62±2%, respectively. Prophylactic treatment with the combination of PER+FLX, however, was not synergistic at preventing adverse LV remodeling. Additionally, the prophylactic administration of FLX, PER, or FLX+PER had no significant effects on serial systolic blood pressure values over the 6 week study. Histological analyses on cardiac tissues samples confirmed significant disruption of myofibrils, vacuolization, and loss of sarcomere integrity in the RC+DOX+TRZ treated mice. Prophylactic administration of FLX or FLX+PER was markedly cardioprotective in preserving myofibril integrity at week 6 in mice receiving the combination therapy of DOX+TRZ. Oxylipin analysis revealed significantly elevated concentrations of inflammatory oxylipins including PGE2 and PGD2 in RC+DOX+TRZ treated mice. Prophylactic administration with FLX, PER, or FLX+PER was able to prevent elevations in inflammatory oxylipins. Finally, western blotting analysis revealed a significant increase in the expression of NF-κβ in RC+DOX+TRZ treated mice. However, pretreatment with FLX, PER, or FLX+PER attenuated elevations in this inflammatory biomarker.

Conclusion: In a chronic *in vivo* female murine model of DOX+TRZ-induced cardiotoxicity, although FLX was equivalent to PER in the prevention of adverse LV remodelling, the combination of FLX and PER was not synergistic.

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In dedication to

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Table of Contents

Abstract ..... **i**

Acknowledgements ..... **iii**

List of Tables ..... **x**

List of Figures ..... **xi**

List of Abbreviations ..... **xiii**

Chapter 1: Introduction **1**

1.1 Breast Cancer: Epidemiology 1

1.2 Breast Cancer: Risk factors, Diagnosis & Treatment Plan 1

1.3 Cardio-Oncology 4

1.4 Chemotherapy 6

1.5 Anthracyclines: Anti-neoplastic properties 8

1.6 The Clinical Use of Doxorubicin 9

1.7 The Human Epidermal Growth Factor Receptors 10

1.8 Breast Cancer and Over Expression of HER2 11

1.9 Trastuzumab and the Inhibition of HER2 11

1.10 The Clinical Use of Trastuzumab 12

1.11 Defining Cardiotoxicity 13

1.12 Doxorubicin-Induced Cardiotoxicity 15

1.13 Trastuzumab-Induced Cardiotoxicity 15

1.14 Prevention of Chemotherapy-Induced Cardiotoxicity 18

1.15 Prevention of Chemotherapy-Induced Cardiotoxicity: RAS Antagonists 19

1.16 Prevention of Chemotherapy-Induced Cardiotoxicity: β-Blockers 21

1.17 Prevention of Chemotherapy-Induced Cardiotoxicity: Anti-Oxidants 22

1.18 Prevention of Chemotherapy-Induced Cardiotoxicity: Statins 23

1.19 Prevention of Chemotherapy-Induced Cardiotoxicity: SGLT2 Inhibitors 24

1.20 Prevention of Chemotherapy-Induced Cardiotoxicity: Nutraceuticals 26

1.20 Flaxseed: Nutritional and Health Benefits 29

1.21 Flaxseed in the Prevention of Chemotherapy-Induced Cardiotoxicity 30

1.22 Treatment of Chemotherapy-Induced Cardiotoxicity 31

1.23 Treatment of Chemotherapy-Induced Cardiotoxicity: RAS Antagonists 32

1.24 Treatment of Chemotherapy-Induced Cardiotoxicity: β-Blockers 33

1.25 Treatment of Chemotherapy-Induced Cardiotoxicity: Anti-Oxidants 34

Chapter 2: Study Rationale, Hypothesis, and Objectives 36

2.1 Study Rationale 36

2.2 Hypothesis 37

Objective 37

Chapter 3: Materials and Methods 38

3.1 Animal Model 38

3.2 Murine Echocardiography 41

3.3 Histological Analysis 44

3.4 Hemodynamics 44

3.5 Oxylipin Analysis 45

3.6 Western Blotting 47

3.7 Statistical Analysis 48

Chapter 4: Results 49

4.1 Murine Echocardiography 49

4.2 Hemodynamics 53

4.3 Histologic analysis 55

4.4 Oxylipin Analysis 57

4.5 Western Blotting 60

Chapter 5: Discussion 62

5.1 Overall Summary 62

5.2 Cardiovascular remodeling 63

5.3 Hemodynamics 66

5.4 Cardiomyocyte injury and mitochondrial disarray 67

5.5 Inflammation 69

5.7 Limitations 72

5.8 Future Directions and Clinical Implications 73

Chapter 6: Conclusion 75

Chapter 7: References 76

List of Tables

Table 1. Molecular features of type I and type II cardiotoxicity. 17

Table 2. Echocardiographic parameters at 6 weeks in C57Bl/6 mice receiving Saline or DOX+TRZ with daily prophylactic treatment of either FLX, PER, or FLX+PER. 50

List of Figures

Figure 1. The cardioprotective potential of FLX and PER in the pathogenesis of DOX+TRZ-mediated cardiotoxicity.[102] 28

Figure 2. Experimental Methodology. 39

Figure 3. Experimental Timeline. 40

Figure 4. Parasternal long axis view on 2D transthoracic echocardiography. 42

Figure 5. M-mode view on 2D transthoracic echocardiography. 43

Figure 6. Changes in LVEDD of mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ. 51

Figure 7. Changes in LVEF of mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ. 52

Figure 8. Changes in MAP of mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ. 54

Figure 9. Cellular alterations in DOX+TRZ-treated mice prophylactically administered FLX and/or PER. 56

Figure 10. Changes in PGE2 concentration in mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ. 58

Figure 11. Changes in PGD2 concentration in mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ. 59

Figure 12. Changes in NF-κβ expression in mice prophylactically treated with FLX, PER, or FLX+PER receiving DOX+TRZ. 61

List of Equations

Equation 1. Left Ventricular Ejection Fraction. 41

Equation 2. Pulse Pressure. 45

Equation 3. Mean Arterial Pressure. 45

List of Abbreviations

AC	Adriamycin - Cyclophosphamide
ACE	Angiotensin converting enzyme
ACEi	Angiotensin converting enzyme inhibitor
ADCC	Antibody-dependent cellular cardiotoxicity
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
ARBs	Angiotensin receptor blockers
Bax	Bcl-2 associated X protein
BNIP3	Bcl-2 interacting protein 3
BP	Blood pressure
β-Blocker	Beta-adrenergic receptor blockers
CCS	Canadian Cardiovascular Society
CMF	Cyclo-phosphamide, methotrexate, and 5-flurorouracil
CMR	Cardiovascular magnetic resonance imaging
COX	Cyclooxygenase
CTRCD	Cancer therapy-related cardiac dysfunction
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DNR	Daunorubicin
DOX	Doxorubicin
DRIs	Direct renin inhibitors
EGFR	Epidermal growth factor receptor

EMPA	Empagliflozin
EPI	Epirubicin
ER	Estrogen receptor
ERD	Estrogen receptor downregulators
FEC	5-Fluorouricil, Epirubicin, and Cyclophosphamide
FLX	Flaxseed
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLS	Global longitudinal strain
HDL	High-density lipoprotein
HER	Human epidermal growth factor receptor
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reducd ejection fraction
HR	Heart rate
IDA	Idarubicin
i.p	Intraperitoneal injection
IVS	Interventricular septal wall thickness
LDL	Low-density lipoprotein
LHRH	Luteinizing hormone releasing hormone
LV	Left ventricle
LVEDD	Left ventricular end-diastolic diameter
LVEF	Left ventricular ejection fraction
LVESD	Left ventricular end-systolic diameter

MAPK	Mitogen-activated protein kinase
MUGA	Multigated acquisition scan
NF-κβ	Nuclear factor kappa B
PARP	Poly (ADP-ribose) polymerase
PLAX	Parasternal long axis
PER	Perindopril
PSAX	Parasternal short axis
RAS	Renin-Angiotensin System
RC	Regular chow
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Standard deviation
SDG	Secoisolariciresinol diglucoside
SEM	Standard error mean
SERMs	Selective estrogen receptor modulators
SGLT2i	Sodium-glucose cotransporter 2 inhibitors
TOPO-II	Topoisomerase II
TRZ	Trastuzumab
T2D	Type 2 diabetes

Chapter 1: Introduction

1.1 Breast Cancer: Epidemiology

According to the Canadian Cancer Society, it is estimated that in 2020, over 110,000 Canadian women will be diagnosed with cancer.[1] In continuation with recent years, breast cancer continues to be the most common cancer in women with 24,700 cases expected.[1] Despite a 49% reduction in mortality since it peaked in 1986, breast cancer remains the second leading cause of cancer related deaths among women.[1] Projected statistics estimate that 1 in 8 women will be diagnosed and 1 in 33 will die from breast cancer in Canada.[1] Nearly 40% of breast cancer diagnoses are in females aged 30 to 59, exemplifying the wide age distribution of breast cancer. The immense economic burden of cancer-related healthcare costs in Canada continue to rise, with breast cancer remaining a leading contributor.[1] Overall, despite the improvement in survival rate for breast cancer patients, the global disease burden continues to rise exemplifying the need for further scientific exploration.[2]

1.2 Breast Cancer: Risk factors, Diagnosis & Treatment Plan

While 12% of the Canadian women will develop breast cancer throughout their lifetime, there are well established risk factors that increases the chances of developing the disease.[1] Heritable factors remain the most potent risk, specifically mutations in the breastr cancer genes (BRCA).[3] BRCA1 and BRCA2 are the breast and ovarian susceptibility genes. Mutations in either gene results in a 80% increased lifetime risk of developing breast cancer.[4] Family history is the second leading risk factor for breast cancer development. As compared to the general population, women with a positive first-degree family history have two-times the risk of developing breast cancer.[1] Other

notable risk factors include atypical hyperplasia, previous chest wall irradiation, increased breast density, late menarche, and hormone replacement therapy.[3]

Reassuring data has shown that breast cancer mortality rates have decreased by 49% since 1986 largely attributed to increased screening and improved treatment options.[1] The Canadian Cancer Society has established screening mammography guidelines suggesting optional screening for women between the ages of 40 – 49 years of age with identified risk factors, and bi-annual screening mammography for women between the ages of 50 – 74.[1] According to the World Health Organization, implementation of such a screening program has been shown to reduce breast cancer mortality by approximately 20%.[5] While mammography plays a vital role in breast cancer screening, core needle biopsies remain the only conclusive diagnostic tool in the breast cancer setting. Microscopic examination of excised tissue not only provides insight into tumor grade, but overall aggressiveness of the neoplasm. Fortunately, the treatment regimens for breast cancer have continued to advance over the years. Surgery, radiation, chemotherapy, and biological therapy remain at the frontline for breast cancer treatment.

Surgical interventions in the treatment of breast cancer was an inaugural first step to improving patient survival in the 1800's. Advancing from radical mastectomies, breast conserving therapies were established to not only lessen the psychological impact but provide a less invasive surgical alternative.[6] While breast conserving therapy soon became the preferred surgical choice for many, it could only be utilized if the breast tumor along with a margin of healthy tissue can be successfully removed. In 2019, a retrospective study of 7565 early breast cancer patients confirmed

that breast conserving therapy followed by radiation therapy resulted in superior local control, distant control, and overall survival as compared to radical mastectomy alone.[7]

Radiotherapy is most commonly employed as an adjuvant treatment following either chemotherapy or surgical intervention in the treatment of women with breast cancer.[1] The addition of radiotherapy following breast conserving therapy has been shown to reduce the recurrence rate by 50% and reduce the breast cancer death rate by approximately 17%.[8] Conversely, radiation therapy can be prescribed prior to surgery as a means to reduce overall tumor size in an effort to increase the success rate of tumor resection. In a retrospective clinical trial, Roth *et al*. (2010) compared the pathologic response, 10-year relapse free, and overall survival of neoadjuvant radiotherapy to adjuvant radiotherapy in a population of locally advanced non-inflammatory breast cancer patients. In summary, neoadjuvant radiotherapy demonstrated superiority in pathologic response, 10-year relapse free, and overall survival when compared to adjuvant radiotherapy, results that were later corroborated by Poleszczuk *et al.* (2017).[9,10]

Hormone receptor status is a key determinant in breast cancer progression, indicative of hormone dependency for neoplastic cell survival and proliferation.[11] Up to 75% of breast cancers are estrogen receptor (ER) status positive, providing unique therapeutic opportunities. Several ER-targeted pharmaceuticals have been synthesized including: Selective estrogen receptor modulators (SERMs), Aromatase inhibitors, Luteinizing hormone-releasing hormone agents (LHRHs), and Estrogen-receptor downregulators (ERDs). SERMs were among the first estrogen receptor targeted therapies to be synthesized for use in the breast cancer setting. SERMs function by blocking the effects of estrogen thereby limiting the signals necessary to grow and multiply.[12]

Conversely, estrogen receptor downregulators (ERDs) trigger the degradation of the estrogen receptor effectively inhibiting hormone binding. The development of aromatase inhibitors, which function by inhibiting the conversion of androgens to estrogens, was of vital importance for postmenopausal women who lack ovarian estrogen production.[13] Lastly, LHRHs function by inhibiting ovarian production of estrogen, and are therefore only effective in pre-menopausal patients whose primary source of estrogen remains ovarian. Although mechanistically independent, all of these drugs work by limiting the bioavailability of the hormone estrogen, thereby impairing ER+ cancer cell survival and proliferation. Interestingly, while most cancers will respond to these drugs, a number of estrogen receptor mutations inhibit their mechanistic action resulting in therapeutic resistance. Novel therapeutics such as bazedoxifene are being explored for their versatility in treating mutated estrogen receptor positive breast cancer.[12]

1.3 Cardio-Oncology

The emerging field of Cardio-Oncology focuses on the prevention, diagnosis, and management of cardiovascular disease in cancer patients. While chemotherapy, radiotherapy, and immunotherapy remain among the most effective cancer treatment modalities, their use is limited due to their inherent cardiotoxicity. Despite marked advances in the treatment of cancer, cardiac dysfunction remains the leading cause of morbidity and mortality among cancer patients.[6] Soon after the advent of anthracycline-based chemotherapy such as Doxorubicin (DOX), cardiotoxicity was recognized as being a detrimental side effect. As a result, the American Society of Echocardiography established cardiovascular guidelines with the goal to standardize the diagnostic criteria of cancer therapy-related cardiac dysfunction (CTRCD). Specifically, CTRCD was defined as a decrease in left ventricular ejection fraction (LVEF) of >10% as compared to baseline to an absolute value of

<53% upon repeat cardiac imaging.[14] Efforts to identify early prognostic criterion remain at the forefront of Cardio-Oncology research. According to the American Society of Echocardiography, a >15% decrease in global longitudinal strain (GLS) is considered subclinical left ventricular systolic dysfunction and has been shown to be a more accurate predictor of CTRCD.[14] Moreover, biomarkers such as cardiac troponins and B-type natriuretic peptide (BNP) are sensitive indices of myocardial damage and are being evaluated for their role in risk stratification and early detection of CTRCD.[15]

Although there are a number of chemotherapeutic agents known to cause cardiac dysfunction, the two most characterized anti-cancer agents for their CTRCD effects include Doxorubicin (DOX) and Trastuzumab (TRZ). Previous studies have established that the incidence of chemotherapy-induced cardiac dysfunction is directly related to the administered cumulative dose of DOX.[16] The average incidence of DOX-mediated cardiotoxicity increases from 5% at a cumulative dose of 400mg/m^2 to 25% at cumulative doses above 550mg/m^2.[16] Adjuvant treatment with TRZ further compounds the cardiotoxic risk, with an incidence ranging from 10-25%.[17,18] While the discernible benefits of chemotherapy, radiotherapy, and immunotherapy have prolonged the lives of many women with breast cancer, their benefits are often attenuated by the adverse cardiovascular side effects.

Determining when to hold anti-cancer treatment to treat progressing cardiac dysfunction takes an integrative, multidisciplinary approach with all the stakeholders involved in the care of Cardio-Oncology patients. Generally, an angiotensin converting enzyme inhibitor (ACEi), such as

perindopril (PER), and beta-blocker (β-Blocker), such as metoprolol, are prescribed to mitigate the cardiovascular complications and prevent further cardiac deterioration.[19]

1.4 Chemotherapy

Developed in the 1950s, the combination of cyclophosphamide, methotrexate, and 5-flurorouracil (CMF) was the first effective chemotherapy regimen for the treatment of breast cancer.[20] Early efforts to improve the efficacy of CMF involved substituting methotrexate for an anthracycline resulting in the successor regimen FEC (5-Flurouracil, Epirubicin, and Cyclophosphamide).[20] Later trials confirmed that the addition of anthracyclines showed superior disease free- and overall patient survival. Currently, the two most common chemotherapeutic regimens used in the treatment of women with breast cancer remain AC (Adriamycin and Cyclophosphamide) and FEC.

AC chemotherapy is a combination of the Adriamycin (A) and Cyclophosphamide (C) that is typically administered biweekly for 6 weeks. In the breast cancer context, AC chemotherapy is typically administered via an intravenous infusion at doses of 60mg/m^2 (A) and 600mg/m^2 (C). Typically, this regimen continues for 3 consecutive cycles unless evidence of disease progression or cardiac dysfunction appears. AC chemotherapy can be used for women presenting with either early or advanced staged breast cancer. AC chemotherapy is often utilized prior to surgery as a neo-adjuvant treatment or following surgery as an adjuvant treatment.

FEC chemotherapy is a combination of 5-Fluorouracil (F), Epirubicin (E), and Cyclophosphamide (C) that is typically administered on a triweekly basis for 12 to 18 weeks. Similar to AC chemotherapy, FEC is administered via an intravenous infusion at cumulative doses of 500mg/m^2,

100mg/m^2, and 500mg/m^2, respectively. Typically, this regimen continues for 4 to 6 consecutive cycles unless certain contraindications emerge. The addition of the microtubule-targeting agent paclitaxel proved advantageous in the breast cancer setting. Specifically, in the GEICAM 9906 study, a total of 1,246 breast cancer patients were randomized to receive either 6 cycles of FEC or 4 cycles of FEC followed by 8 weeks of paclitaxel (100mg/m^2/wk).[21] Individuals administered paclitaxel following FEC chemotherapy showed a 23% reduction in risk of relapse and a 22% reduction in the risk of death when compared to FEC alone.[21]

Immunotherapeutic treatment options for human epidermal growth factor receptor 2 (HER2) positive breast cancer has continued to advance from the original monoclonal antibody, TRZ. While the advent of TRZ revolutionized the treatment of women with HER2+ positive breast cancer, disease progression remained evident. In a landmark clinical trial, Slamon *et al*. (2001) evaluated the efficacy of adjuvant TRZ therapy with DOX in metastatic breast cancer patients.[22] Overall, it was shown that combination therapy with TRZ resulted in higher response rates, longer duration of response, and reduced risk of death as compared to DOX monotherapy.[22] Since then, several targeted therapies have transformed the treatment of HER2+ breast cancer including pertuzumab, lapatinib, and most recently ado-trastuzumab emtansine. Fortunately, early preclinical and clinical studies have demonstrated these emerging target therapies to be less cardiotoxic.[23–25] Ado-trastuzumab emtansine functions by conjugating a TRZ covalently to the anti-mitotic agent maytansine allowing for targeted delivery of the cytotoxic agent directly to HER2+ cancer cells.[26] Although mechanistically independent, all HER2 target immunotherapies work to counteract the enhanced cell survival induced by the HER2 pathway. New immunotherapeutic technologies such as oncolytic virus therapies are being explored for their

therapeutic efficacy in treating breast cancer.[27] Induction of a robust systemic anti-cancer immune response by viral particles can result in a death of tumor cells which may be used in conjunction with current cytotoxic therapies to enhance overall treatment.[27]

1.5 Anthracyclines: Anti-neoplastic properties

Isolated from *Streptomyces peucetius* in the 1960s, DOX and daunorubicin (DNR) soon became the gold standard adjuvant therapy for breast cancer.[28,29] In an effort to improve the therapeutic indices of DOX and DNR, semi-synthetic derivates such as Epirubicin (EPI) and Idarubicin (IDA) were soon developed. While these second-generation analogs have resulted in improved therapeutic indices through their pharmacodynamic advances, the risk of cardiomyopathy is not eradicated.[29] Anthracyclines exhibit their anti-neoplastic actions through a wide number of mechanisms including: 1) DNA intercalation resulting in inhibited DNA synthesis; 2) generation of free radicals resulting in DNA damage and lipid peroxidation; 3) DNA binding and alkylation; 4) DNA cross-linking; 5) interference with DNA unwinding or DNA strand separation; 6) direct membrane damage; 7) DNA damage resulting from inhibition of topoisomerase II (Topo-II); and/or 8) induction of apoptosis.[29,30] Importantly, a number of these mechanisms were only reported *in vitro* at concentrations significantly higher than those used clinically.[29] Accordingly, the most widely accepted clinically accepted anti-neoplastic mechanisms of anthracyclines include generation of free radicals and inhibition of topoisomerase II.[29]

Generation of reactive oxygen species (ROS) results from a series of futile redox cycling reactions. A one-electron addition to the DOX structure results in a semiquinone free radical. In aerobic conditions, the semiquinone free radical reduces O_2 producing an unstable superoxide anion. This

superoxide anion causes a sequelae of detrimental intracellular effects including the induction of single or double stranded DNA breaks, and lipid peroxidation.[29,30] Combined, these macromolecular modifications result in cellular apoptosis justifying its vital role in the anti-tumor properties of anthracyclines.

Topo-II is a critical enzyme responsible for modifying the topological state of DNA during replication and transcription. Responsible for inducing and resealing transient single and double stranded DNA breaks to limit supercoiling, Topo-II is pivotal in regulating normal cell cycle and transcriptional activity. Through intercalation with DNA, DOX forms a cleavable complex hindering its enzymatic activity. This global DNA damage result in the activation of the p53 pathways, ultimately inhibiting cell proliferation and inducing apoptosis.[29]

1.6 The Clinical Use of Doxorubicin

Doxorubicin is routinely used in the clinic as a chemotherapeutic agent for the treatment of various cancers including both solid and hematologic malignancies.[29,31] However, the utility of DOX is limited by its cumulative, dose-dependent cardiotoxicity. Thus, considerable efforts need to be made in order to detect, prevent, and treat progressive cardiac dysfunction in this patient population. Identifying risk factors for DOX-induced cardiotoxicity is of vital importance as it provides information to help clinicians choose between different cumulative doses.[31,32] The current risk factors for DOX-induced cardiotoxicity include cumulative dose, age, and pre-existing cardiovascular risk factors including obesity, hypertension, and diabetes.[31] While treatment with a cardiotoxic drug such as DOX may prolong survival for women with breast cancer, it is essential to be aware of the associated risk of cardiotoxicity. As such, a maximum cumulative dose of

500mg/m^2 during an individual's lifetime has been established to lessen the risk of these adverse side effects.[16] In an effort to improve the efficacy of DOX, while limiting its cardiotoxicity, a liposomal formulation was developed. A process known as pegylation encapsulates DOX in phospholipid bilayer with surface-bound methoxypolyethylene glycol.[33] Prolonged systemic circulation and selective DOX delivery to malignant cells are two of the proposed mechanism for the superior therapeutic index of liposomal DOX.[34] However, unanimity has not been reached that liposomal formulations improve overall survival in breast cancer patients.[35,36]

1.7 The Human Epidermal Growth Factor Receptors

Growth factors are essential for the development, growth, and homeostasis of multicellular organisms.[37] The human epidermal growth factor receptors (ErbB/HER/c-*neu*) are a group of proteins from the receptor tyrosine kinases (RTK) family, responsible for initiating intracellular signal transduction pathways for cellular proliferation, apoptosis, and angiogenesis.[37] The ErbB subset of RTK consists 4 closely related isoforms: HER1, HER2, HER3, and HER4. Each specific transmembrane HER receptor is activated by extracellular ligand binding and receptor dimerization. Interestingly, HER2 is the only isoform not activated via extracellular ligand binding, but rather acts as a common receptor through heterodimerization with all other isoforms. Once dimerized, subsequent activation of both the Ras-Raf-Mitogen-Activated protein kinase (Ras-Raf-MAPK) and phosphatidylinositol 3-kinase/protein kinase B pathways to enhance cell survival and proliferation.

1.8 Breast Cancer and Over Expression of HER2

Indicative of a poor clinical prognosis, up to 30% of women with breast cancer over express the HER2 (ErbB2) receptor isoform.[38] Unfortunately, the elevated aggressiveness associated with HER2+ breast cancer has been shown to interfere with the efficacy of the current breast cancer treatment armamentarium. Amplification of the HER2 gene results in elevated numbers of HER2 surface receptor proteins leading to enhanced cell proliferation, survival, and angiogenesis. Normally, breast cells express low levels of HER2 protein that maintain basal cell survival and proliferative effects. However, oncogenic transformation results in amplified expression leading to uncontrollable cell division and enhanced neoplastic growth.[39] Clinically, early HER2 status determination is advantageous as prompt anti-HER2 treatment improves patient prognosis. HER2 status is determined by carrying out Immunohistochemistry (IHC) or Fluorescent *in situ* hybridization (FISH) on neoplastic tissue. IHC results are interpreted to be negative, equivocal, or positive, depending on the pattern and degree of staining. Specifically, tumors with absent or weak staining (<30% tumor cells) are indicative of normal HER2 gene status. Results exhibiting uniform, intense membrane staining in >30% of tumor cells are considered positive, whereas, heterogeneity in cellular staining is to be considered unequivocal and require additional FISH testing.[40] Historically, HER2 status had tremendous prognostic significance, however, with the advent of anti-HER2 therapies patient outcomes have drastically improved.[23]

1.9 Trastuzumab and the Inhibition of HER2

Trastuzumab (TRZ), a monoclonal antibody against the extracellular domain of the HER2 receptor, has become a mainstay for HER2-positive breast cancer treatment. Approved in 1998, TRZ shows favorable treatment outcomes for HER2-positive patients when used as both a single

and adjuvant agent.[41] The addition of TRZ showed sustained benefit in event-free survival and overall response rate when administered in conjunction with FEC combination therapy.[21] When bound, TRZ mechanistically exhibits it anti-tumor properties by preventing heterodimerization of HER isoforms, promoting receptor internalization, and degradation.[41,42] Additionally, supporting evidence suggests a potential role in inducing antibody-dependent cellular cardiotoxicity (ADCC). Specifically, ADCC is induced when the exposed Fc region of the bound TRZ is recognized by immune effector cells causing cellular degradation.[41] Collectively, TRZ therapy effectively curtails the cascade of biochemical and physiological growth signals exhibited by HER2 overexpression, resulting in reduced neoplastic cell resilience.

1.10 The Clinical Use of Trastuzumab

The addition of targeted immunotherapy, namely TRZ, in the treatment of breast cancer proved influential in the HER2-positive breast cancer setting. The FDA approved TRZ for use in both the adjuvant and metastatic settings of breast cancer. The HERA trial sought to investigate the long-term disease outcomes of HER2+ early breast cancer patients treated with surgery, chemotherapy, and/or radiotherapy randomized to receive adjuvant TRZ treatment for 1 year.[43] Overall, adjuvant TRZ treatment demonstrated superiority in long-term disease-free survival, and mortality at a median follow-up of 11 years.[43] While the cardiotoxic effects of anthracyclines alone were previously reported, a multicenter, randomized control trial sought to investigate the cardiotoxic effects of anthracyclines with and without TRZ therapy. Adjuvant TRZ therapy resulted in cardiac dysfunction in up to 27% of HER2-positive metastatic breast cancer patients as compared with only 7% in the anthracycline only arm.[22]

1.11 Defining Cardiotoxicity

In 2014, the American Society of Echocardiography and European Association of Cardiovascular Imaging, defined CTRCD as a decrease in the LVEF of >10% from baseline, to an absolute value of less than 53%.[14] In cases where cardiotoxicity is discovered, repeat cardiac imaging needs to be performed 2 to 3 weeks following the initial examination to not only corroborate the initial findings, but also deduce information regarding reversibility.[14] Cardiotoxicity is considered reversible if the LVEF improves 5 percentage points from baseline, partially reversible if the LVEF improves by ≥10 percentage points from the nadir but remains >5 percentage points from the baseline value, and irreversible if the LVEF improves <10 percentage points from the nadir and remains >5 percentage points from the baseline value.[14] It is evident that early detection of CTRCD is critical for preventing detrimental long-term cardiovascular effects in breast cancer survivors, and thus efforts to elucidate novel sensitive indices are warranted.

While cardiac MRI (CMR) is currently considered the gold standard for quantification of LVEF, echocardiography is the modality of choice for serial cardiac monitoring of patients before, during, and after cancer therapy due to its availability, repeatability, and versatility.[14] Despite their widespread use, each of these imaging modalities have shortcomings that limiting the detection of chemotherapy-induced cardiotoxicity. Early detection with high sensitivity may prompt earlier treatment modifications thereby reducing the incidence of severe CTRCD in cancer patients. Emerging parameters derived from speckle-tracking echocardiography, such as GLS, have proved advantageous in the early detection of CTRCD. In a 2019 observational study, Narain *et al*. (2019) concluded that 2D GLS detected myocardial dysfunction resulting from chemotherapy earlier than conventional systolic and diastolic measures.[44] While many established Cardio-Oncology

guidelines have incorporated LV-GLS in the determination of subclinical LV systolic dysfunction, emerging evidence suggests RV systolic function may be a promising prognostic indicator as well.[45] In a recent study, Keramida *et al*. (2019) investigated the prognostic capabilities of RV myocardial strain in breast cancer patients who developed cardiomyopathy as a result of their anti-cancer therapy.[46] In this study, impairment of RV GLS directly corresponded to impairments in LV GLS at 6 month follow-up. Additionally, Keramida *et al*. (2019) found that the absolute cut-off value for RV GLS that predicted chemotherapy-induced cardiotoxicity was strikingly similar to the Canadian Cardiovascular society (CCS) established LV GLS cut-off (14.8% vs 15%).[46] Overall, further studies are warranted to determine if biventricular assessment would provide superior detection of chemotherapy-induced cardiotoxicity.

Nuclear imaging has historically been the cornerstone for the detection of cardiotoxicity accompanied with oncologic treatment. The use of Multigated acquisition scan (MUGA) scans in Cardio-Oncology patients has drastically declined largely due to the excess radiation exposure associated with its use. Data estimates radation exposure associated with each MUGA study is equivalent to approximately 10-15 chest X-rays.[47] Additionally, MUGA scans do not provide any anatomical or functional information about RV function or atrial sizes and cannot detect valvular or pericardial diseases.[14] Despite the wide availability and general cost-effectiveness of MUGA scans, new echocardiographic and CMR technologies are the preferred imaging modalities for serial monitoring of LVEF in Cardio-Oncology patients. When discordant imaging findings arise or echocardiographic evaluation is limited due to patient's body habitus, CMR is an effective alternative.

1.12 Doxorubicin-Induced Cardiotoxicity

Despite its broad-spectrum anti-neoplastic capabilities, the clinical use of DOX is often limited by its dose-dependent cardiotoxicity. Approaches to reduce the cardiotoxic side effects continue to galvanize research in the Cardio-Oncology field. Studies investigating the rate and duration of DOX administration, as well as novel liposomal analogs have provided valuable insight in the clinical development of DOX-induced cardiotoxicity but have not proved clinically useful.[48,49]

Classified as type I cardiotoxicity, DOX is believed to cause a surge of free radical production and oxidative stress resulting in programmed cardiomyocyte death. This has led to the notion that DOX causes dose-dependent, irreversible cardiac dysfunction (Table 1).[14,16] In a recent study, Caballero *et al*. (2020) evaluated the incidence of long-term cardiotoxicity in women with breast cancer treated with DOX. A total of 85 patients receiving a mean DOX dose of 254mg/m^2 were enrolled.[50] Echocardiographic parameters were measured in all patients at baseline and various time points up to 4.5 years following start of chemotherapy. Overall, the incidence of long-term cardiotoxicity exhibited by DOX was 15% (14 of 85 total patients). The majority of these patients presented with asymptomatic systolic dysfunction (12 of 14), with the remaining two suffering from heart failure (HF) (1 of 14) and sudden death (1 of 14).[50]

1.13 Trastuzumab-Induced Cardiotoxicity

While the monoclonal antibody TRZ improves the oncologic outcomes among HER2+ breast cancer patients, its use is often associated with untoward cardiotoxic side effects.[51,52] When used as monotherapy, the incidence of TRZ-induced cardiotoxicity ranges between 3-7%.[52] The addition of anthracycline therapy further compounds the cardiotoxic risk of TRZ with a combined

incidence of up to 25%.[51] In contrast to anthracyclines, TRZ exhibits type II toxicity resulting in dose-independent, reversible cardiomyocyte damage (Table 1).[14,53,54] Discontinuing TRZ therapy often results in reversal of cardiac dysfunction, with TRZ rechallenging occurring only if sufficient cardiac recovery has occurred.[54] Molecularly, TRZ competitively binds to the extracellular domain of the HER2 surface receptors effectively downregulating the vital cell survival pathways. As a result, TRZ therapy can elevate cardiomyocyte stress by several known mechanisms. TRZ inhibits autophagy leading to an accumulation of ROS effectively damaging cellular macromolecules including: proteins, nucleic acids, and lipids. Additionally, TRZ causes a downregulation of Topoisomerase-II, and dysregulation of sarcomeric organization leading to profound cellular alterations.[54,55]

In a recent study, Laird-Fick *et al*. (2020) investigated the ultrastructural changes in cardiac tissue in rabbits treated with subcutaneous TRZ.[56] In their study, administration of TRZ resulted in several ultrastructural cardiomyocyte changes including: i) immune cell infiltration; ii) myofibril disruption & sarcomere rupture; and ii) impaired mitochondrial cristae.[56] Interestingly, ultrastructural changes directly correlated with cumulative dosage of TRZ administration and were significantly attenuated when animals were supplemented with the oral antioxidant selenium.[56]

Table 1. Molecular features of type I and type II cardiotoxicity.

Type 1 Cardiotoxicity (ex. DOX)	Type 2 Cardiotoxicity (ex. TRZ)
Cardiomyocyte death	Cardiomyocyte damage
Cumulative dose-related	Not dose-related
Irreversible	Reversible
Dose-Dependent	Dose-Independent
Risk factors: Combinational chemotherapy Prior or concomitant radiation therapy Age Previous cardiac disease HTN	Risk factors: Prior or concomitant anthracyclines Prior or concomitant paclitaxel Age Previous cardiac disease Obesity (BMI > 25kg/m^2)

BMI, Body mass index; DOX, Doxorubicin; HTN, Hypertension; TRZ, Trastuzumab.

1.14 Prevention of Chemotherapy-Induced Cardiotoxicity

The lack of adequate treatment options for patients who develop chemotherapy-induced cardiac dysfunction has spurred the discovery of novel *preventative* strategies. Renin-angiotensin system (RAS) antagonists, β-blockers, anti-oxidants, and most recently, nutraceuticals, are all potential cardioprotective agents studied in the prevention of chemotherapy-induced cardiotoxicity. Despite the lack of established clinical consensus surrounding the use of prophylactic therapies, cardioprotective agents, such as these, may be an effective strategy at improving overall patient survival. Currently, the CCS recommend the use of either: i) ACE inhibitor; ii) angiotensin receptor blocker; iii) β-blockers; and/or iv) statin in the management of cardiovascular complications associated with anti-cancer therapies.[57] Depending on several factors including degree of cardiac dysfunction, stage of cancer, and treatment outcome goals (curative vs. palliative), an unfortunate reality is that initiation of a cardiac preserving medication is often accompanied by cessation of vital anti-cancer therapy.

Dexrazoxane, an iron-chelating agent approved by the FDA for cardioprotective use in 1995 continues to capture the attention of scientists and clinicians. Originally proposed to be universally prescribed to those receiving a cardiotoxic agent, concerns over increased risk of infection, reduced anti-cancer response rates, and elevated risk of secondary malignancies soon became apparent.[58] In a multicenter clinical trial, women with breast cancer receiving an anthracycline based chemotherapy were randomized to receive either placebo or dexrazoxane. Adverse cardiac events were defined as a LVEF decrease of >10% from baseline. The hazard ratio of placebo to dexrazoxane for cardiac events was 2.0 (95% confidence interval of 1.01 to 3.96, $p = 0.0038$) illustrating its efficacy in preventing cardiac damage. However, dexrazoxane treatment resulted in

a 14% decreased anti-cancer response rate as compared to control (95% confidence interval of -25% to -2%, p = 0.019).[58] Studies such as this have led to more stringent recommendations, particularly, limiting the use of dexrazoxane only to women with advanced metastatic breast cancer who receive more than 300 mg/m^2 doxorubicin or 540 mg/m^2 epirubicin.[58–60]

The grim prognosis faced by Cardio-Oncology patients presents an indisputable need to discover novel prevention and remedial treatment strategies. Although numerous cardioprotective agents have been identified including RAS antagonist, β-blockers, anti-oxidants, and nutraceuticals, high-caliber clinical trials are lacking thereby limiting their clinical impact.

1.15 Prevention of Chemotherapy-Induced Cardiotoxicity: RAS Antagonists

Increased understanding of the RAS revealed a significant contribution to the pathogenesis of many cardiovascular diseases. Beginning in the 1970s, scientific inquiry surrounding the potential therapeutic target of the RAS in cardiovascular disease began.[61] The development of angiotensin converting enzyme inhibitors (ACEi), followed by the angiotensin receptor blockers (ARBs), and most recently direct renin inhibitors (DRIs) proved instrumental for cardiovascular medicine.[61,62] Collectively, all RAS antagonists work by decreasing the bioavailability of the peptide hormone angiotensin II. Angiotensin II has been shown to promote atherogenesis through combined effects on muscle cell proliferation and migration, immune cell activation, vascular invasion, increased oxidative stress and stimulation of thrombosis.[63,64] Early studies investigated the effects of the ACEi enalapril on survival in patients with reduced LV ejection fraction and congestive HF. As compared to control, treatment with enalapril resulted in a 16% reduction in overall mortality.[65]

The cardioprotective effects of RAS antagonists in the setting of chemotherapy-induced cardiac dysfunction has captured much interested and thus various basic science and clinical trials have been completed. Akolkar *et al*. (2015) reported the cardioprotective effects of the DRI aliskiren, ACEi PER, and ARB valsartan in a preclinical model of DOX+TRZ-induced cardiac dysfunction. Specifically, a total of 240 mice were randomized to 13 weeks of prophylactic treatment with: i) placebo; ii) DRI aliskiren; iii) ACEi PER; or iv) ARB valsartan. Mice were then further randomized to receive DOX, TRZ, or DOX+TRZ. Interestingly, prophylactic treatment with all three RAS antagonists prevented adverse cardiac remodeling, and preserved fractional shortening values.[66] Moreover, in the MANTICORE study, Pituskin *et al*. (2017) investigated the cardioprotective effects of both the ACEi PER and β-Blocker bisoprolol in breast cancer patients receiving adjuvant TRZ treatment.[67] Overall, it was shown that treatment with the β-blocker bisoprolol lessened LVEF impairment when compared to the ACEi PER.[67] The Prevention of Cardiac Dysfunction During Adjuvant Breast Cancer Therapy (PRADA) study, conducted by Heck *et al*. (2018) investigated the cardioprotective effects of the ARB candesartan and β-blocker metoprolol in breast cancer patients receiving FEC chemotherapy. Overall, it was shown that the ARB candesartan was able to partially preserve LVEF when compared to β-blocker metoprolol.[68]

Finally, in a 2020 meta-analysis of 17 randomized control trials, Avila *et al*. (2020) evaluated the efficacy of RAS antagonists and β-Blockers in mitigating anthracycline induced changes in LVEF and the incidence of HF in adults undergoing anthracycline chemotherapy.[69] Overall, use of both RAS antagonists and β-Blockers for prevention of anthracycline-cardiotoxicity was associated with preserved LVEF and a lower incidence of symptomatic HF; however, no change in mortality were observed in this meta-analysis.[69]

1.16 Prevention of Chemotherapy-Induced Cardiotoxicity: β-Blockers

Elevated sympathetic nervous system activation resulting in neurohormonal alterations is a well-established contributor to the pathogenesis of cardiovascular disease. Excessive neurohormonal activation results in hemodynamic alterations, adverse cardiac remodeling, arrhythmias, and desensitization of the beta-adrenergic signal transduction pathways.[70] As such, the advent of beta-adrenergic receptor blockers (β-Blockers) resulted in reduced mortality and improved overall functioning in cardiac patients.[71] Overall, β-Blockers can be either selective, effectively inhibiting sympathetic nerve stimulation of β1 receptors exclusively, or non-selective, resulting in inhibition of β1 and β2 receptors. Stimulation of β1 receptors present within cardiac tissues result in positive inotropic (contractility) and chronotropic (heart rate) effects. In contrast, stimulation of β2 receptors present in the vasculature results in smooth muscle relaxation resulting in vasodilation.[72]

A number of basic science and clinical trials have investigated the efficacy of β-Blockers in preventing chemotherapy-induced cardiotoxicity. The CECCY trial, conducted by Avila *et al*. (2018) randomized 200 HER2-negative breast cancer patients to receive the β-Blocker carvedilol or placebo in addition to their anthracycline chemotherapy.[73] The primary endpoint used was a ≥10% reduction in LVEF at 6 months. In the carvedilol group, the primary endpoint was reached in 14 patients as compared to 13 patients in the placebo group. Therefore, the use of β-blockers did not convey significant cardioprotection as compared to control.[73] Moreover, in a 2019 multicenter, randomized control trial, Guglin *et al*. (2019) examined the effects of lisinopril and carvedilol in preventing anthracycline and TRZ-induced cardiotoxicity in 468 HER2-positive breast cancer patients.[74] In patients treated with both TRZ and anthracyclines, lisinopril and

carvedilol both resulted in a lower incidence of cardiotoxicity ($p<0.05$). Additionally, both treatment interventions resulted in fewer TRZ interruptions as compared to the placebo control ($p=0.011$).[74]

Additionally, in a 2020 meta-analysis of 11 randomized control trials, Xu *et al*. (2020) evaluated the cardioprotective effects and duration of β-Blocker therapy on anthracycline-induced cardiotoxicity.[75] Overall, prophylactic β-Blocker use was associated with statistically significant improvements in systolic function. Additionally, cardiotoxic risk was significantly lower in patients treated with β-Blockers for 6 months when compared to those treated <6 months ($p<0.05$).[75] Collectively, given the inconsistency and scarcity of evidence surrounding the potential cardioprotective effects of β-Blockers in mitigating chemotherapy-induced cardiotoxicity, further clinical investigations are warranted.

1.17 Prevention of Chemotherapy-Induced Cardiotoxicity: Anti-Oxidants

Experimental evidence strongly suggests the role of reactive oxygen species (ROS) in the process of DOX-mediated cardiotoxicity.[28,29] While the use of anti-oxidants may be an effective strategy at mitigating the cardiotoxicity associated with chemotherapy use, controversies exist over whether anti-oxidants will attenuate the cytotoxic properties of anti-cancer agents.[76] Several basic science studies have investigated the role of probucol, vitamin C, catechin, superoxide dismutase (SOD), and N-acetylcysteine amide (NACA) in preventing cardiac toxicity in a preclinical model of chemotherapy-induced cardiac dysfunction.[77–81] Collectively, these basic science findings concluded that anti-oxidant supplementation can attenuate the cardiotoxic side effects associated

with chemotherapy use. Additionally, the utility of anti-oxidant supplementation in the clinical setting of chemotherapy-induced cardiotoxicity has proven promising. In a recent study, Ambrosone *et al*. (2019) investigated the effect of anti-oxidant supplementation on modulating the cytotoxic properties of anti-cancer agents in the setting of breast cancer. Overall, anti-oxidant supplementation either before or during chemotherapy resulted in an increased rate of recurrence with an adjusted hazard ratio of 1.41 (95% CI 0.98 – 2.04), and death with an adjusted hazard ratio of 1.40 (95% CI 0.64 – 0.86).[82] Lastly, in a meta-analysis of 33 randomized control trials, Block *et al*. (2008) sought to investigate the impact of antioxidant supplementation on chemotherapeutic toxicity. They found that the majority of studies (24/33) reported evidence of reduced cardiotoxicity associated with antioxidant supplementation and only one (1/33) study reporting increased toxicity.[83] However, with such conflicting basic science evidence surrounding the potential benefits of anti-oxidant supplementation in reducing the toxicity associated with chemotherapy, further basic science and clinical trials are needed.

1.18 Prevention of Chemotherapy-Induced Cardiotoxicity: Statins

Statins, also known as 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA) reductase inhibitors, are a widely prescribed class of lipid lowering medications used primarily in the management of atherosclerotic cardiovascular disease. Largely due to their anti-inflammatory and anti-oxidative properties, statins have been studied in both basic science and clinical studies for their role in preventing anthracycline-induced cardiac dysfunction. Contradictory to RAS antagonists and β-Blockers which result in depressed blood pressure and heart rate (HR), repectively, leading to fatigue, statins may be a favourable alternative in both the prevention and treatment of chemotherapy-induced cardiac dysfunction. Riad *et al*. (2009) investigated the effects

of fluvastatin pre-treatment on doxorubicin-induced cardiotoxicity in a murine model. Pre-treatment with fluvastatin resulted in enhanced cardiac function as demonstrated by improved LV pressures and overall cardiac output.[84] Additionally, Henninger e*t al*. (2015) investigated the effects of lovastatin on anthracycline-induced late cardiotoxicity in a murine model. Interestingly, while decreases in left ventricular posterior wall diameter were prevented by lovastatin administration, fractional shortening and LVEF were not preserved by lovastatin treatment.[85]

Several clinical trials have corroborated these early basic science findings. Specifically, Calvillo-Argüelles *et al*. (2019) investigated the cardioprotective effects of statin treatment in patients with HER2-positive breast cancer receiving adjuvant TRZ therapy. In this retrospective study, statin treatment was associated with improved systolic function and reduced overall cardiotoxicity when compared to control.[86] It is noteworthy that women treated with statins were more likely to have diabetes, hypertension, and coronary artery disease. Finally, in a recent prospective randomized control trial, Nabati *et al*. (2019) investigated the effect of rosuvastatin treatment in preventing chemotherapy-induced cardiotoxicity in 84 breast cancer patients.[87] Patients were randomized in a 1:1 ratio for 6 months to rosuvastatin or placebo. Overall, patients randomized to placebo experienced a considerable drop in LVEF while those subject to 6 months rosuvastatin had preserved LVEF.[87] Additionally, in contrast to those randomized to rosuvastatin, there was a significant increase in 4- and 2-chamber LVESV experienced in control patients.[87]

1.19 Prevention of Chemotherapy-Induced Cardiotoxicity: SGLT2 Inhibitors

Sodium-glucose cotransporter-2 inhibitors (SGLT2i) are a class of drugs originally used in combination with diet and exercise to lower blood glucose levels in adults with type-2 diabetes

(T2D).[88] Incidences of T2D have doubled since 1980 and appear to remain on the rise.[89] Additionally, T2D is associated with cardiovascular complications including: LV hypertrophy, endothelial dysfunction, and cardiac fibrsosis.[88] As a result, T2D and HF often coexist with more severe patient outcomes, extended hospital stays, and heightened clinical management costs.[90] In the landmark EMPEROR-REDUCED Trial, Packer *et al*. (2020) demonstrated the superiority of the SGLGT2i empagliflozin (EMPA) vs. placebo in reducing the risk of cardiovascular mortality or hospitalization for HF regardless of the presence or absence of T2D.[91] As a result, EMPA became the first diabetic medication to be approved for use in CV death protection in T2D patients. The promising results demonstrated in the EMPEROR-REDUCED trial have made scientists and clinicians question whether SGLT2i have utility in the prevention and management of HF resulting from anti-cancer therapy.

In a preclinical study, Chang *et al*. (2021) investigated the cardioprotective potential of dapagliflozin in the setting of DOX-induced cardiotoxicity.[92] Using a rat model of DOX-induced cardiotoxicity, they found that oral administration of 10mg/kg/day dapagliflozin for 6-weeks resulted in improved LVEF and fractional shortening parameters, reduced LV fibrosis, and reduced cardiomyocyte death.[92] Similarly, Oh *et al*. (2019) demonstrated the cardioprotective capabilies of EMPA in a murine model of DOX-induced cardiotoxicity.[93] In summary, they found that 0.03% EMPA-supplemented diet preserved fractional shortening, reduced oxidative stress, and ameliorated interstitiaul and perivascular fibrosis associated in mice administered DOX.[93] Lastly, Sabatino *et al*. (2020) investigated the effects of EMPA in preventing DOX-induced myocardial dysfunction.[94] In summarly, mice were randomized to either control, DOX, or the combination of DOX+EMPA and monitored via echocardiography for a total of 5 weeks. At the

study endpoint, mice treated with DOX had significantly reduced LVEF when compared to control with values of 49.2±8 and 68.7±5%, respectively.[94] Supplementation with 10mg/kg/day EMPA was able to preserve systolic function with a LVEF of 61.3±11%.[94] Similarly, supplementation with EMPA was able to preserve longitudinal and circumferential strain parameters, reduce cardiac fibrosis, and attenuate elevations in serum biomarkers associated with DOX-induced cardiotoxicity.[94] These early preclinical investigations have demonstrated that the prophylactic administration with an SGLT2i may prevent the adverse cardiotoxic side effects of anti-cancer therapy due to anthracyclines.

1.20 Prevention of Chemotherapy-Induced Cardiotoxicity: Nutraceuticals

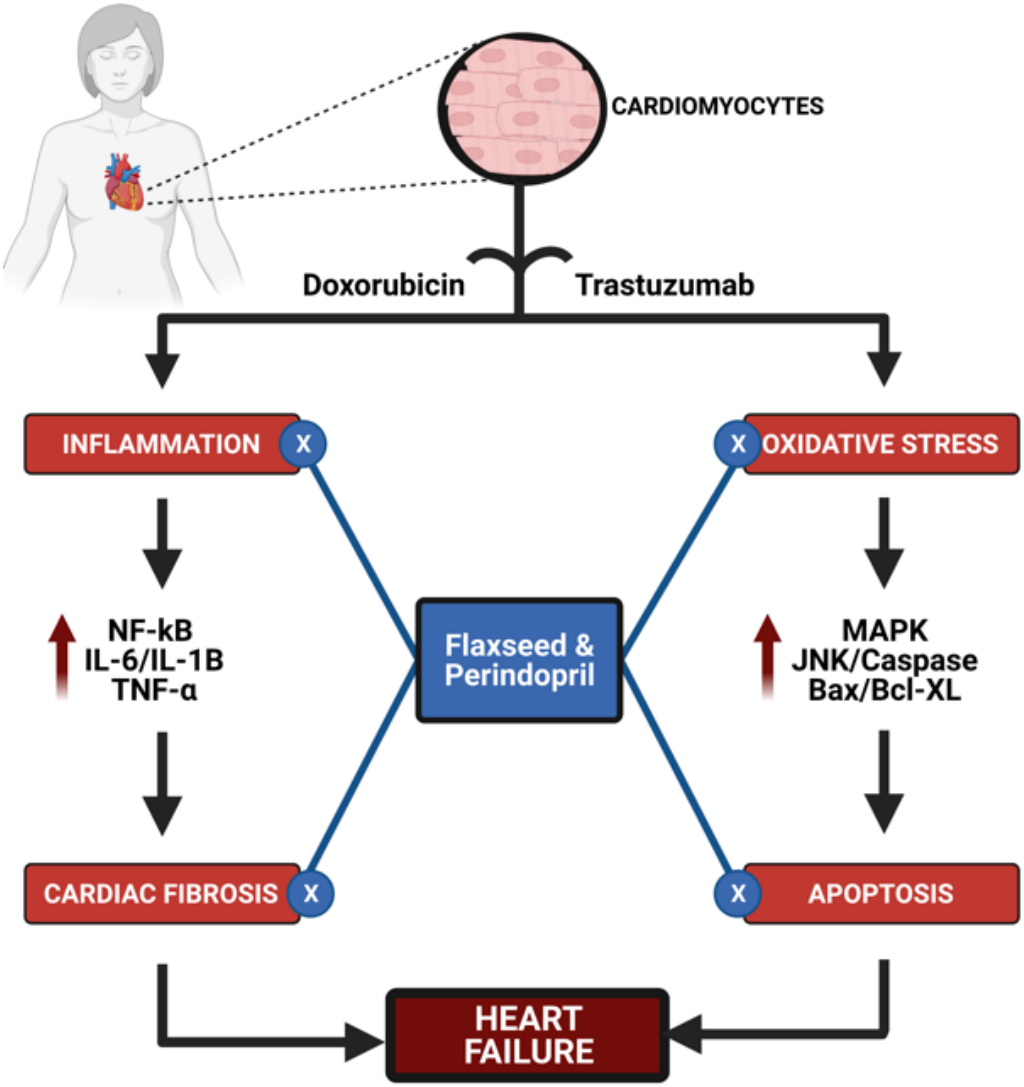
Nutraceuticals can be broadly defined as a functional food that aid in the prevention, management, and treatment of a disease or disorder.[95,96] Despite the many existing dietary nutraceuticals including ginseng, echinacea, and vitamin E, discovering novel cardioprotective nutraceuticals remains at the forefront of Cardio-Oncology research.

Recent studies investigating the effect of sulforaphane, a naturally occurring phytochemical found in cruciferous vegetables in preventing DOX-induced cardiotoxicity has captured scientific attention. These studies have illustrated the cardioprotective effects of sulforaphane in both *in vitro* and *in vivo* breast cancer models.[97,98] Supplementation with sulforaphane during DOX treatment not only prevented DOX-induced cardiomyopathy and oxidative stress in rat H9c2 cardiomyoblast cells but also protected mice against DOX-induced declines in LVEF. Additionally, sulforaphane potentiated the anti-neoplastic effects of DOX resulting in improved tumour regression in tumor bearing-rats.[97] Similarly, Hijazi *et al*. (2019) demonstrated the cardioprotective effects of the

Achillea fragrantissima plant extract in preventing DOX-induced cardiotoxicity in rats.[99] Supplementation with *Achillea fragrantissima* plant extract protected the animal models from DOX-induced electrophysiological ST-segment elevation as well as DOX-induced histopathologies including necrosis and perivascular edema of coronary vessels. Hijazi *et al.* proposed the mechanism of protection includes anti-inflammatory and anti-oxidative pathways.[99]

Consumption of FLX has been shown to be advantageous in a variety of conditions including cardiovascular and cancer pathologies.[100] FLX has captured the attention of several Cardio-Oncologists for its potential role in preventing chemotherapy-induced cardiotoxicity (Figure 1). In a 17-day experimental study performed in rats, Yu *et al*. (2013) investigated the cardioprotective effects of FLXs bioactive constituent alpha-linolenic acid (ALA) on DOX-induced cardiotoxicity. When compared to control, co-treatment with ALA resulted in enhanced systolic function as measured by LVEF and reduced expression of apoptotic markers. Moreover, while DOX treatment resulted in a downregulation of SOD, co-treatment with ALA was able to preserve this natural cellular antioxidant.[101]

Figure 1. The cardioprotective potential of FLX and PER in the pathogenesis of DOX+TRZ-mediated cardiotoxicity.[102]



In a *in vivo* chronic female murine model of DOX+TRZ-mediated cardiotoxicity, FLX and PER are able to attenuate inflammation, oxidative stress, apoptosis, and LV cardiac fibrosis associated with DOX+TRZ administration.[66,102] Bax, Bcl-2-associated X protein; Bcl, B-cell lymphoma 2; IL-1B, Interleukin-1B; IL-6, Interleukin-6, JNK, c-Jun N-terminal kinases; MAPK, Mitogen-activated protein kinase; NF-kB, Nuclear factor kappa-B; TNF-a, Tumor necrosis factor alpha.

1.20 Flaxseed: Nutritional and Health Benefits

Emerging evidence continues to support the use of FLX in a variety of health conditions, including diabetes, cardiovascular disease, and cancer.[103–107] Broadly, it is proposed that FLX exhibits it physiological benefits through anti-inflammatory, anti-oxidative, and anti-atherosclerotic effects.[100] The main bioactive components found in FLX include ALA, and the lignan secoisolariciresinol diglucoside (SDG). While ALA is responsible for potent anti-inflammatory properties, SDG possesses anti-atherosclerotic and anti-oxidative qualities.[100,108]

Several studies have sought to investigate the effects of FLX supplementation on cancer risk and progression, most notably in the setting of breast cancer.[106,107,109] In both experimental animal studies and human trials, supplementation with dietary FLX provided marked protection against breast cancer.[106,107,109] Additionally, FLX consumption results in reduced disease progression and tumor growth in breast cancer patients.[106,107] As a result, up to 30% of breast cancer patients consume FLX to reduce disease progression and prevent worsening co-morbidities.[110,111] Hu *et al*. (2019), investigated the *in vitro* effects of FLX extract on cell growth and apoptosis in human MCF-7 breast cancer cells. FLX extract resulted in increased apoptosis of MCF-7 cancer cells as shown by flow cytometric analysis.[112] Moreover, in a recent randomized phase IIB trial, Fabian *et al*. (2020) investigated the effect of SDG supplementation on cell proliferation in benign breast tissue of premenopausal women at risk for development of breast cancer. The primary endpoint was differences in Ki-67 expression (indicative of increased cell growth and proliferation) between SDG and placebo randomization groups. Overall, although supplementation with SDG resulted in a median Ki-67 change of -1.8% (P=0.001). Uniquely, a similar reduction in Ki-67 expression was seen in the placebo arm (-1.2%, P=0.034) resulting in no statistical significance.[113] The promising

data surrounding the use of FLX in the prevention and treatment of breast cancer not only proves significant public health importance, but calls for additional investigation.

In the context of cardiovascular disease, supplementation with FLX has been associated with hypotensive, lipid-lowering, antiarrhythmic, and anti-atherosclerotic properties.[100,114–116] In a recent meta-analysis, Askarpour *et al*. (2020) showed that FLX supplementation resulted in reduced circulating concentrations of adhesion molecules and inflammatory cytokines commonly associated with endothelial dysfunction and cardiovascular disease.[117] Similarly, Hadi *et al*. (2020) analyzed the lipid modulating effects of FLX of 62 randomized control trials. Overall, FLX supplementation resulted in reduced serum triglyceride, low-density lipoprotein (LDL) cholesterol, and total cholesterol levels while leaving high density lipoprotein (HDL) cholesterol levels unchanged.[118] Considering the corroborated evidence surrounding the many health benefits of FLX in the setting of a variety of conditions including cardiovascular disease, its role as a dietary therapy in the clinical setting warrants further investigation.

1.21 Flaxseed in the Prevention of Chemotherapy-Induced Cardiotoxicity

While the cardiotoxic mechanism associated with chemotherapy use is inconclusive, up-regulation of inflammatory mediators and oxidative stress remain two well established pathways. As FLX exhibits anti-inflammatory and anti-oxidative properties through its bioactive components, ALA and SDG, it may prove to be a promising candidate in prevention of chemotherapy-induced cardiac dysfunction. In a recent basic science study performed by our lab, Asselin *et al*. (2020), illustrated the cardioprotective effects of FLX, ALA, and SDG in a chronic *in vivo* female murine model of DOX+TRZ mediated cardiotoxicity.[102] In this study, mice treated with RC+DOX+TRZ

demonstrated a decrease in LVEF from 75±2% at baseline to 37±3% at week 6 ($p<0.05$). Prophylactic administration of FLX, ALA, and SDG partially attenuated LV systolic function with LVEF values of 62±2%, 61±3%, and 62±4% respectively ($p<0.05$).[102] In mice treated with RC+DOX+TRZ, there was an approximate 2-fold increase in markers of inflammation, apoptosis, and mitochondrial dysfunction as compared to control. Prophylactic administration of FLX, ALA, and SDG downregulated these pathological signaling pathways ($P<0.05$).[102] Finally, histological examination revealed that supplementation with FLX, ALA, and SDG abated DOX+TRZ-induced loss of cellular integrity and myofibril disarray when compared to control ($p<0.019$, $p<0.033$, and $p<0.002$, respectively).[102] Given the impressive evidence supporting the role of FLX in preventing chemotherapy-induced cardiotoxicity, additional studies are warranted to not only evaluate whether FLX can recover function following the induction of DOX+TRZ cardiomyopathy, but compare the effects of FLX to cardioprotective pharmaceuticals such as ACEi in this setting.

1.22 Treatment of Chemotherapy-Induced Cardiotoxicity

While cardioprotective measures should be considered for all patient's subject to a cardiotoxic chemotherapy regimen, cardiac dysfunction may still develop. As a result, several guidelines and position statements have been established to provide clinical consensus in treating the cardiovascular consequences associated with chemotherapy use.[14,57,119] According to the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines, treatment for patients who develop LV systolic dysfunction during anthracycline chemotherapy should be consistent with the treatment for HF.[120,121] Generally, an ACEi such as PER and/or a β-Blocker, such as Metoprolol, are prescribed to mitigate the cardiovascular complications and prevent further cardiac deterioration.[19] Additionally, careful decisions regarding cessation of anti-

cancer therapies need to be made ensuring best patient prognosis. Considerations for chemotherapy withdrawal include: i) severity of cardiotoxicity; ii) clinical burden of HF; iii) the cancer prognosis; and/or iv) availability of a less cardiotoxic anti-cancer regimen.[122] Resumption of anti-cancer therapies are generally contingent on sufficient recovery of cardiac functioning.[14,57,122] However, an unfortunate reality is that 0.5-2.5% of CTRCD patients advance into end-stage HF with cardiac transplantation remaining the only viable treatment option.[123]

1.23 Treatment of Chemotherapy-Induced Cardiotoxicity: RAS Antagonists

The CCS guidelines recommend the use of ACEi/ARBs and β-Blocker in symptomatic patients or in asymptomatic patients experiencing a decline in LVEF of ≥10% from baseline to an absolute value of <53%.[14,57] Although numerous clinical trials have demonstrated the efficacy of RAS antagonists in treating clinical cardiac dysfunction, data is limited surrounding the use of RAS antagonists in recovering cardiac function in the setting of chemotherapy-induced cardiotoxicity.

In a prospective clinical trial, Cardinale *et al*. (2010) investigated the efficacy of the ACEi enalapril and carvedilol in treating anthracycline induced cardiomyopathy.[124] Overall, patients were considered responders when LVEF increased to an absolute value of ≥50%, partial responders when LVEF increased at least 10 percentage points but not to an absolute value of ≥50%, and nonresponders when LVEF increased fewer than 10 percentage points and did not reach an absolute value of ≥50%.[124] Of the 201 patients enrolled, 42% were responders (85/201), 13% partial responders (26/201), and 45% nonresponders (90/201) to ACEi treatment. Additionally, the best predictor for responsiveness was early initiation of ACEi therapy, exemplifying the importance of early detection strategies.[124] In a larger prospective clinical trial performed by the

same group, treatment with either enalapril resulted in either full (11%), or partial (70%) LVEF recovery following chemotherapy-induced cardiac dysfunction.[125]

1.24 Treatment of Chemotherapy-Induced Cardiotoxicity: β-Blockers

While the use of β-Blockers constitutes the mainstay treatment for a variety of cardiac indications including hypertension, dysrhythmias, and HF, its use in treating chemotherapy-induced cardiotoxicity remains inconclusive.[126] According to the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines, β-Blockers are recommended for patients who develop HF with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF) without contraindications such as bradycardia or hypotension.[121,125]

In a retrospective clinical trial, Ohtani *et al*. (2019) investigated whether treatment with ACEi and β-Blockers would be superior at recovering cardiac function following anthracycline induced cardiotoxicity when compared to ACEi or β-Blockers alone.[127] In summary, treatment with concurrent ACEi and β-Blocker therapy provided superior improvement in LV systolic function when compared to either intervention alone. Additionally, concordant with previous studies, the best predictor of treatment success was timely initiation of HF medication.[127] As previously mentioned, prompt initation of HF medication including either enlapril or enalapril and β-Blockers resulted in either full (11%), or partial (70%) LVEF recovery following chemotherapy-induced cardiac dysfunction.[125] Despite the paucity of data surrounding the potential remedial benefits of β-Blockers in the setting of chemotherapy-induced cardiotoxicity, further studies are warrented.

1.25 Treatment of Chemotherapy-Induced Cardiotoxicity: Anti-Oxidants

Elevated concentrations of ROS has been a well established contributor to a variety of health conditions including arthritis, amyloidosis, and atherosclerosis.[128] As a result, antioxidants such as ascorbic acid, and allopurinol have been synthesized and used to treat a variety of physiological indications.[128,129] Specific to Cardio-Oncology, there is a paucity of clinical data surrounding the potential role of anti-oxidant supplementation in recovering cardiac functioning. However, several *in vitro* studies have occurred with the goal of elucidating the molecular effects of antioxidant treatment on cardiomyocyte dysfunction.

Ellagic acid, a naturally occurring phenol antioxidant has recently been studied for its potential cardiorecovery effects in the setting of DOX-induced cardiotoxicity. Dhingra *et al*. (2017) sought to investigate the effects of ellagic acid treatment on Bnip3-mediated mitochondrial injury and necrotic cell death in rat cardiomyocytes.[130] Briefly, rat cardiomyocytes were treated DOX followed by ellagic acid at doses of 10μM and 1-20μM respectively, and examined for biochemical indices of cardiotoxicity. Overall, treatment with ellagic acid was associated with markedly reduced mitochondrial associated Bnip3 concentrations in cell treated with DOX.[130] Similarly, treatment with ellagic acid suppressed DOX-mediated necrotic cell death and mitochondrial injury.[130]

In a similar study, Ibrahim *et al*. (2017) investigated both the antioxidant and antiapoptotic effects of sea cucumber and valsartan in rats induced with DOX-mediated cardiotoxicity.[131] In short, rats were treated with six equal injections of DOX (2.5mg/kg, i.p.) followed by 8 successive weeks of sea cucumber (14.4mg/kg, orally) and/or valsartan (30mg/kg, orally). Treatment with DOX alone

resulted in elevated serum concentrations of lactate dehydrogenase (LDH), creatine kinase, and troponin indicative of elevated cardiomyocyte stress as well as evidence of histopathological findings.[131] Consumption of sea cucumber and valsartan resulted in improved cardiotoxicity as measured by reduced cardiotoxic serum biomarkers, inflammatory and apoptotic markers, and histopathological findings.[131] Interestingly, combinational therapy did not provide superior cardioprotection when compared to sea cucumber and valsartan monotherapy.[131] Impressive data such as these illumiate the importance of continuing scientific inquiry surrounding the potential role of dietary antioxidants in mitigating the cardiotoxicity associated with chemotherapy use.

Chapter 2: Study Rationale, Hypothesis, and Objectives

2.1 Study Rationale

While the combination of DOX+TRZ is highly effective at reducing morbidity and mortality in women with breast cancer, their benefits are significantly attenuated by cardiotoxic side effects. Up to 1 in 4 breast cancer patients receiving the combination of DOX and TRZ may develop cardiotoxicity.[17,51] As a result, novel strategies to prevent and treat chemotherapy-induced cardiotoxicity remain at the forefront of Cardio-Oncology research.

Current guidelines by the CCS recommend patients at high risk for chemotherapy-induced cardiotoxicity be prescribed an RAS antagonist and/or β-Blocker to reduce the cardiotoxic hazard.[57] Similarly, once cardiac dysfunction has developed due to chemotherapy, an ACEi such as PER and/or β-Blocker are recommended to mitigate the cardiovascular complications and prevent further cardiac deterioration.[57]

Scientific evidence supporting the use of dietary FLX in a variety of health conditions continues to grow.[100] FLX has been shown to not only prevent development but reduce tumor progression in breast cancer patients.[106] Additionally, our lab recently demonstrated the cardioprotective effects of FLX in a chronic *in vivo* murine model of chemotherapy-induced cardiotoxicity.[102] Little is known, however, whether the administration of FLX is equivalent and/or syngerstic with PER in the prevention of chemotherapy-induced cardiotoxicity.

2.2 Hypothesis

The cardioprotective effects of dietary FLX will be equivalent and/or synergistic with conventional PER at preventing cardiac dysfunction in an *in vivo* murine model of DOX+TRZ-induced cardiomyopathy.

Objective

To determine whether the prophylactic administration of FLX will be comparable and/or synergistic with PER at preventing adverse cardiovascular remodeling in a chronic *in vivo* murine model of DOX+TRZ-induced cardiomyopathy.

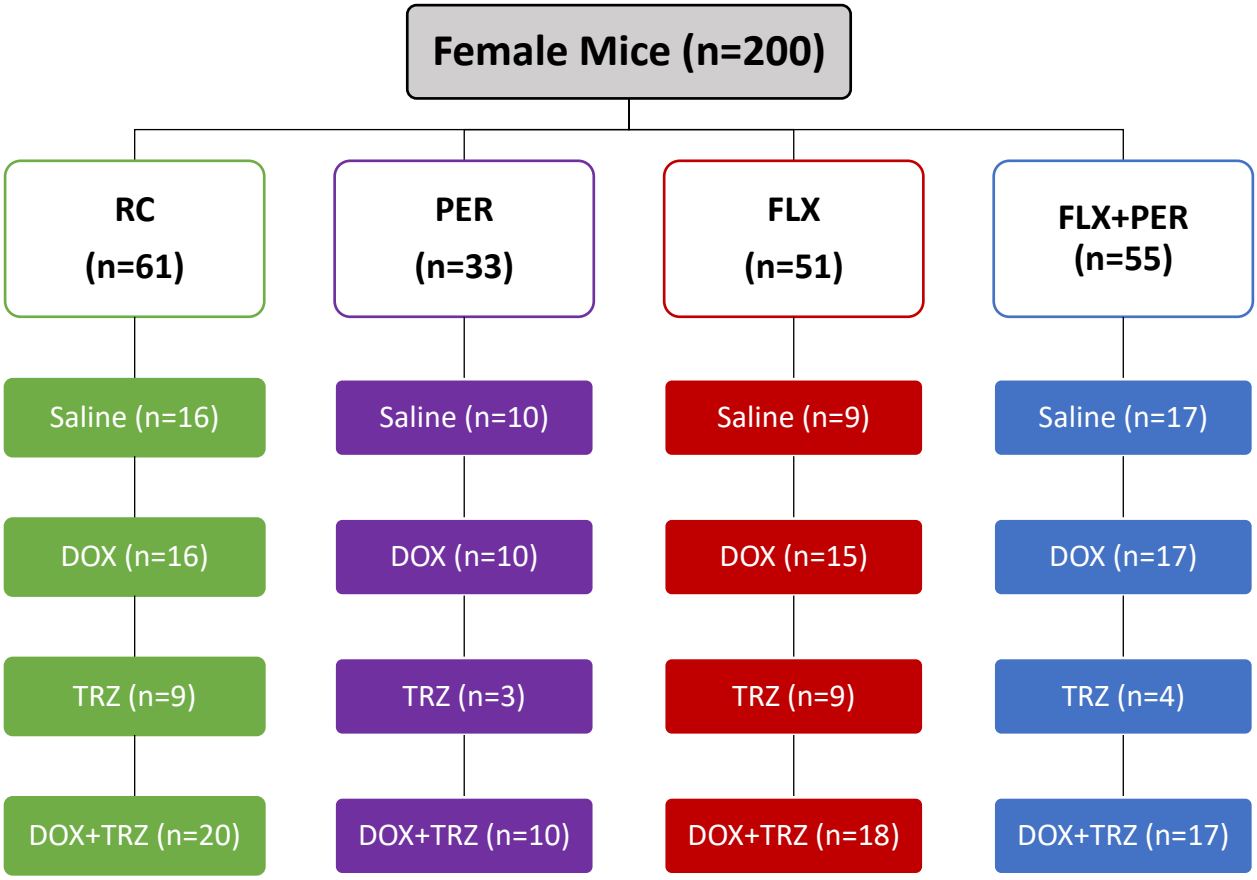
Chapter 3: Materials and Methods

3.1 Animal Model

All animal procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care. The Animal Protocol Review Committee at the University of Manitoba approved all procedures, including drug administration and longitudinal echocardiographic studies [REB: 17-022/3 (AC11285)].

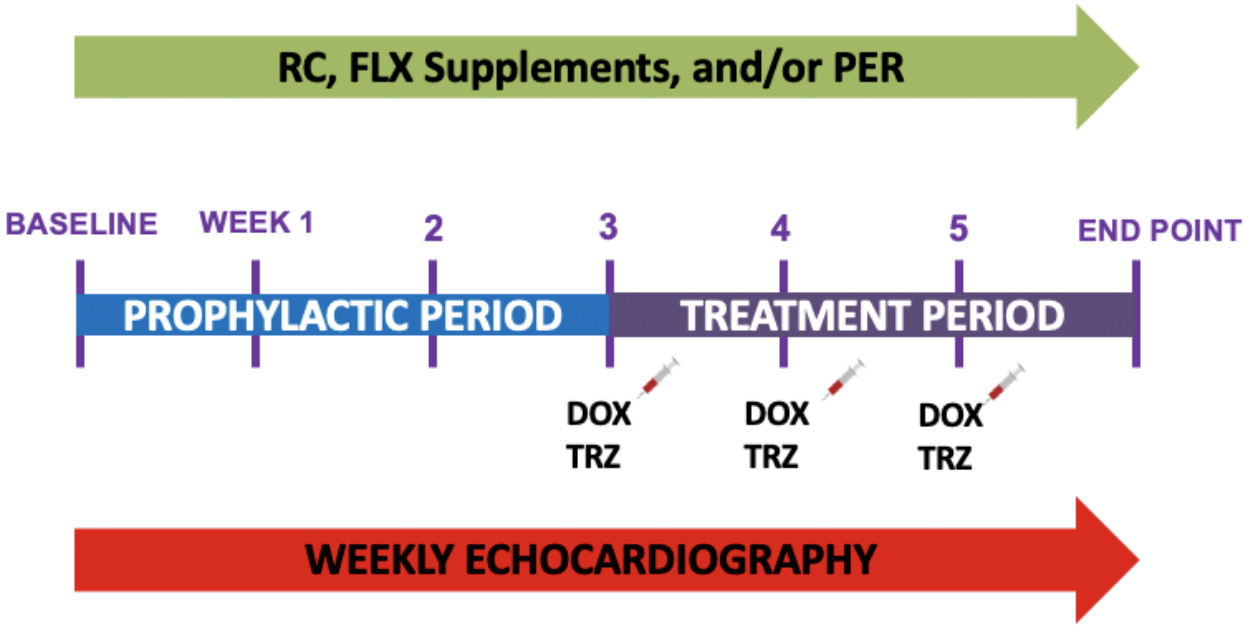
A total of 200 wild-type C57B1/6 female mice (8-12 weeks old; Jackson Laboratories, Bar Harbor, ME, US) were quarantined for 1 week prior to the initiation of the study. The specific sample size was selected to allow for sufficient statistical power (>85%) to detect a difference of 0.5 standard deviation as significant. All animals were maintained on a 12-hour day/night cycle and received *ad libitum* access to the study diets as well as water during their stay in the animal holding facility. All mice were subject to echocardiograms, hemodynamics, and weight analyses prior to initiation of study diets. All mice were randomized to receive to receive either: i) Regular Chow (n=94); or ii) 10% FLX (n=106) supplemented diets with or without PER (3mg/kg) (Figure 2). The cardioprotective effects of both 10% FLX and 3mg/kg PER were validated by our lab.[66,132]Additionally, excluding the bioactive components, the nutritional composition are comparable between RC and FLX. Finally, mice were further randomized to receive one of four i.p injections of: i) 0.9% Saline; ii) DOX (8mg/kg)[133]; iii) TRZ (3mg/kg)[133] ; or iv) DOX+TRZ (8mg/kg and 3 mg/kg, respectively)[133] on weeks 4, 5, and 6. (Figure 3)

Figure 2. Experimental Methodology.



A total of 200 WT C57Bl/6 female mice (8-10 weeks old; Jackson Laboratories, ME, US) were randomized into one of four dietary groups including: RC (n=61); PER (n=33); FLX (n=51); or FLX+PER (n=55). Mice received *ad libitum* access to their respective diets for the entire 6-week study. At weeks 4,5 and 6, mice were further randomized to receive an intraperitoneal injection of 0.9% saline, DOX (8mg/kg)[133], TRZ (3mg/kg)[133], or DOX+TRZ in order to induce a chronic *in vivo* model of chemotherapy-induced cardiotoxicity. DOX, doxorubicin; FLX, flaxseed; PER, perindopril; RC, regular chow; TRZ, trastuzumab.

Figure 3. Experimental Timeline.



Mice received *ad libitum* access to their respective diets on a daily basis for the entirety of the 6-week study. At the start of weeks 4,5 and 6, mice were further randomized to receive an intraperitoneal injection of 0.9% saline, DOX (8mg/kg)[133], TRZ (3mg/kg)[133], or DOX+TRZ in order to induce a chronic state of chemotherapy-induced cardiotoxicity. Cardiac function was assessed weekly using non-invasive echocardiography. Hemodynamic parameters were measured at baseline, week 3 and 6. At study endpoint, cardiac tissues were harvested for both histological and biochemical analysis. DOX, doxorubicin; FLX, flaxseed; PER, perindopril; RC, regular chow; TRZ, trastuzumab.

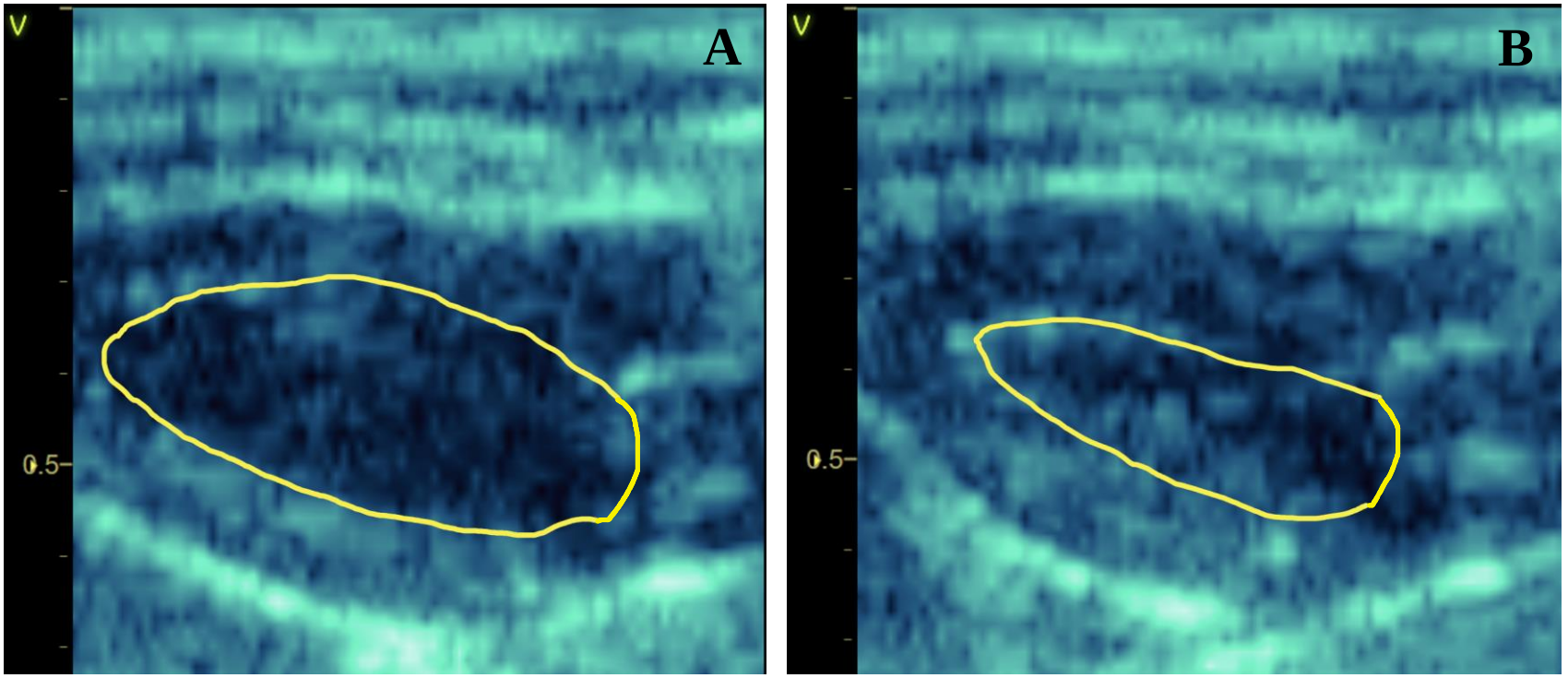
3.2 Murine Echocardiography

Cardiac function was assessed in all mice using non-invasive transthoracic echocardiography at baseline and weekly for the entirety of the 6-week study. A 13-MHz linear array ultrasound probe (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US) was used to capture parasternal long axis (PLAX), parasternal short axis (PSAX), and M-mode views on all awake mice as previously described (Figure 4 & 5).[80,134] Post-acquisition analyses were performed on all images, and endocardial borders determined for LVEF calculation (Equation 1). Echocardiographic-derived measurements include: i) LV end-diastolic diameter (LVEDD); ii) LV end-systolic diameter (LVESD); iii) posterior wall thickness (PWT); and iv) interventricular septal wall thickness (IVS). The EchoPAC PC software (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US) was used for offline post-processing of all images and calculation of LVEF.

Equation 1. Left Ventricular Ejection Fraction.

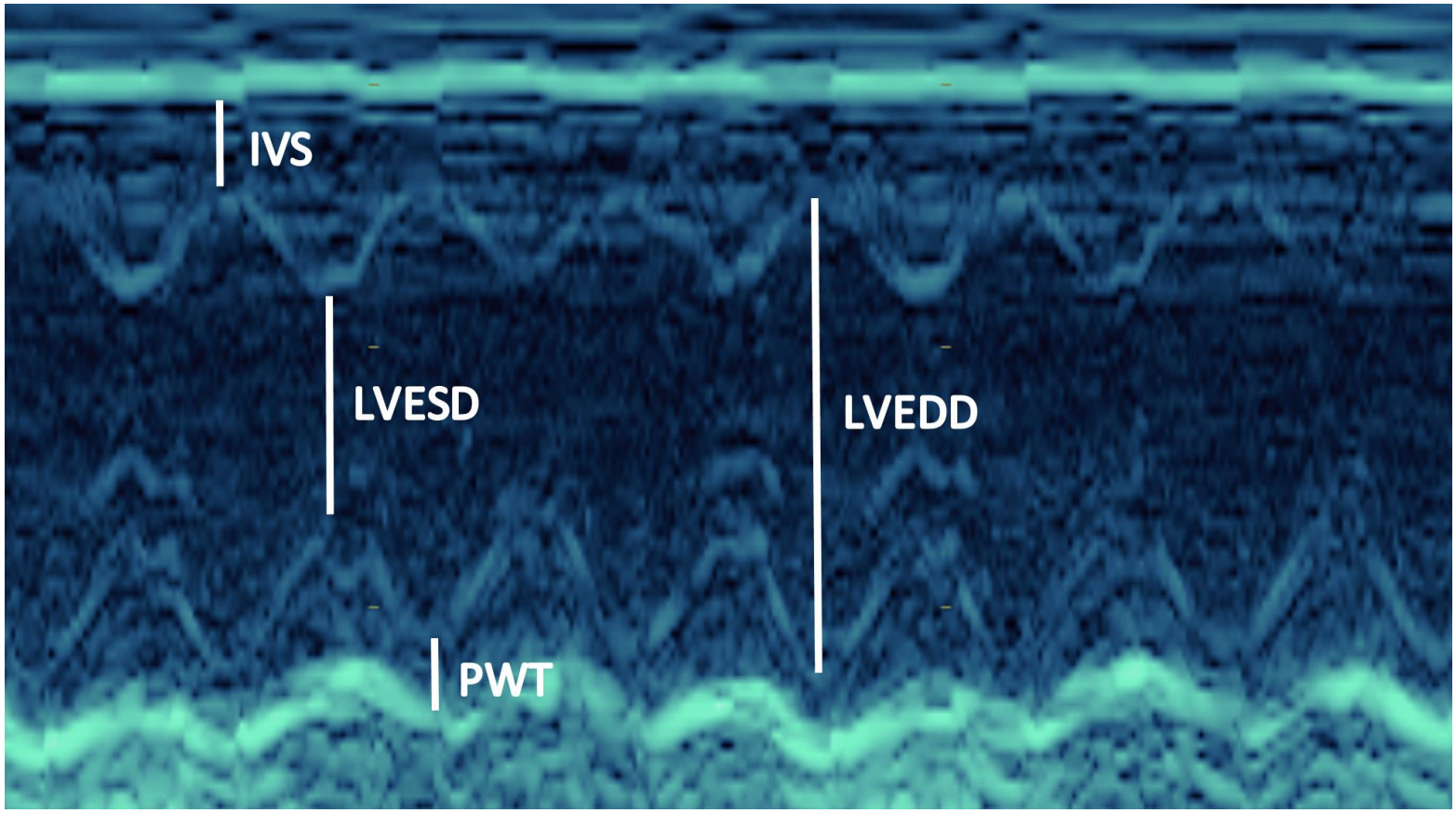
$$\text{LVEF} = \frac{(\text{LV end diastolic volume} - \text{LV end systolic volume})}{\text{LV end diastolic volume}} \text{ x } 100$$

Figure 4. Parasternal long axis view on 2D transthoracic echocardiography.



LV endocardial border delineation on EchoPAC PC software (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US) in order to calculate LVEF. **Panel A:** Endocardial border tracing at end diastole. **Panel B:** Endocardial border tracing at end systole. LV, Left ventricle; LVEF, Left ventricular ejection fraction.

Figure 5. M-mode view on 2D transthoracic echocardiography.



LV cavity dimensions as measured using M-mode on EchoPAC PC software (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US). IVS, interventricular septum; LVEDD, left ventricular end-diastolic diameter ; LVESD, left ventricular end-systolic diameter; PWT, posterior wall thickness.

3.3 Histological Analysis

Cardiac tissue samples were placed in 3% glutaraldehyde in 0.1M phosphate buffer at pH 7.3 to fix the tissues for 3 hours in preparation for examination using the electron microscope. Tissues were then rinsed in 0.1M phosphate buffer containing 5% sucrose overnight at 4°C. Post fixation was then performed with 1% osmium tetroxide in 0.1M phosphate buffer for 2 hours at room temperature. Tissues were dehydrated in ascending ethanol concentrations and embedded in Epon 812 as previously described.[135] After the tissue sections were stained with uranyl acetate and lead citrate, they were viewed and photographed with the Philips CM12 electron microscope in order to determine the degree of cellular integrity. To avoid observer bias, grids were coded without prior knowledge of their source. For histological analysis, Mann-Whitney and Kruskal-Wallis tests were applied for non-parametric comparison of scores between each group. The scores ranged from 1 to 4, with 1 representing no tissue injury and 4 representing severe damage.

3.4 Hemodynamics

Using a non-invasive tail cuff method (CODA system, High Throughput, Kent Scientific, Torrington, CT) hemodynamics measurements including systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) were recorded in restrained, non-sedated mice. Hemodynamics were measured at baseline, week 3, and at study endpoint. The holding platform was heated to 30°C, and 15 successive blood pressure (BP) readings were recorded with a 1-minute break period between readings. The mean scores of 15 true blood pressure readings were included in the data set. Pulse pressures were calculated from the diastolic and systolic pressure values using Equation 2. Pulse pressures were then used in Equation 3 to calculate MAP of each mouse. The average values for SBP were computed using 9 individual readings.

Equation 2. Pulse Pressure.

$$\text{Pulse Pressure} = \text{LV Systolic Pressure} - \text{LV Diastolic Pressure}$$

Equation 3. Mean Arterial Pressure.

$$\text{Mean Arterial Pressure} = \frac{Pulse\ Pressure}{3} + \text{Diasolic Pressure}$$

3.5 Oxylipin Analysis

A total of 100μL of plasma was added to a 2mL Eppendorf tube containing 1mL pH 3.0 water and 100μL of internal standard. Samples were vortexed, and pH tested using indicator strips. If needed, samples were acidified to pH 3.0 using approximately 5 μL of 1N hydrochloric acid (HCl). If the pH dropped below 3.0, 1N sodium hydroxide (NaOH) was used to raise the pH back to 3.0. Excess debris was removed by centrifuging the samples for 10 minutes at 14000 RCF at 4°C. Strata-X SPE (Phenomenex, 33μ, 60 mg/3 mL) columns for each sample were arranged on a wooden rack and used to extract the oxylipin metabolites. Columns were conditioned using 3.5mL of MeOH, followed by equilibration by flushing column with 3.5mL pH 3.0 water using a 10 mL syringe. Samples were then added to the corresponding column. A total of 1 mL of 10% MeOH in pH 3.0 water was added to vial, vortexed and then centrifuged at 14000 RCF at 4°C for 5 minutes to collect any remaining sample that could be added to the column. Each column was washed with 2 mL of pH 3.0 water and dried by pushing 1 mL of Hexane. A 1.5 mL microtube was placed underneath each column once completely dried. Each column was eluted using 1 mL MeOH whereby pressure was applied to allow MeOH to soak the column for 1 minute. MeOH was then

pushed through in order to collect the samples. Excess air was displaced using Nitrogen gas (N2) before the samples were vortexed, spun down, and stored at -20°C.

Frozen microtubes containing the samples were thawed and placed into the evaporator set to 37°C. Needles were lowered to allow N2 gas to gently blow at the surface of the samples. The samples were left to dry for 60 to 90 minutes. Once dry, 100 μL of cold solvent A (water/acetonitrile/formic acid, 70/30/0.02 v/v/v) was immediately added to the dried samples, which were then vortexed and centrifuged at 14000 RCF at 4°C for 10 minutes. The samples were then transferred into labelled GC/LC vials, which contained a 200 μL polypropylene conical insert. Once the samples were eluted with 100% Methanol, dried down under N2, and reconstituted in the mobile phase (water/acetonitrile/formic acid, 70/30/0.02 v/v/v), the supernatant was transferred into a labelled GC/LC vial containing a 200 μL polypropylene conical insert and analyzed by high-performance liquid chromatography-electrospray ionization-mass spectroscopy, as described by Deems *et al*. (2007) Briefly, all MS analyses were performed using an Applied Biosystems (Foster City, CA) 4000 QTRAP hybrid, triple-quadrupole, linear ion trap mass spectrometer equipped with a Turbo V ion source and operated in MRM. The Turbo V ion source was operated in negative electrospray mode and the QTRAP was set as follows: CUR = 10 psi, GS1 = 30 psi, GS2 = 30 psi, IS = -4500 V, CAD = HIGH, TEM = 525°, ihe = ON, EP = -10 V, and CXP = -10 V.393 The voltage used for CID (-15 to -35 V) and thedelustering potentials (-30 to -100 V) varied according to molecular species.

3.6 Western Blotting

In the presence of liquid nitrogen, frozen heart tissue was ground into a powder and homogenized in radioimmunoprecipitation (RIPA) buffer to extract total levels of protein. The RIPA buffer is composed of 50 mM Tris pH 7.4, 150 mM Sodium Chloride (NaCl), 1 mM EDTA, 1 mM EGTA, 0.5% Na-deoxycholate, 1% Triton-X 100, and 0.1% sodium dodecyl sulfate (SDS). Additionally, protease (Product #: A32965) and phosphatase (Product #: PIA32957) inhibitors (Thermo Scientific) were added to the RIPA buffer prior to its use to prevent protein degradation. After the lysates were incubated on ice for 1 hour and centrifuged at 14000 RPM for 10 minutes at 4°C, the supernatants were collected. Total protein concentration was measured using the Bradford assay which included the Coomassie Blue Protein Assay Reagent (Product #: 1856209, Thermo Scientific) and bovine serum albumin (BSA) standards (Product #: 23209, Thermo Scientific). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was employed at 55mA for 90 minutes to separate 30 μg of protein. The proteins were then transferred to a 0.2 μm pore size polyvinylidene fluoride membrane (Product #: 88520, Thermo Scientific) using 100 V for 60 minutes at 10°C. Using 5% skim milk powder or BSA in 1X Tris Buffered Saline with 0.1% Tween 20, the membranes were blocked for 60 minutes at room temperature. The membranes were probed overnight at 4°C with primary antibodies specific to poly (ADP-ribose) polymerase (PARP), Bcl-2 associated X protein (Bax), B-cell lymphoma extra-large (Bcl-XL), Nuclear factor kappa B (NF-κβ), phospho-NF-κβ, Bcl-2 interacting protein 3 (Bnip3) and Caspase (Product #: 9542S; 2772S; 2762S; 8242S; 3031S; 3769S; 9662S, New England Biolab). Primary antibodies specific to the glyeraldehyde 3-phosphate dehydrogenase (GAPDH) loading control was added and left to incubate for 1 hour. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody

(BioRad) was added to the membrane and left to probe for 60 minutes. Protein detection was accomplished using Pierce ECL Western Blotting Substrate (Product #: 32106, Thermo Scientific) on CL-Xposure blue X-ray film (Product #: XC6A2, Mandel Scientific Company Inc.). Protein band intensity was quantified by Densitometric analysis using QuantityOne software (BioRad) normalized to the loading control GAPDH.

3.7 Statistical Analysis

All data are expressed as mean ± standard deviation (SD). For Western analysis, the data are expressed as mean ± standard error mean (SEM). For post hoc analysis, repeated measures of one-way analysis of variance (ANOVA) were used to evaluate for significance between independent factors. P values for main effects and interactions were noted when appropriate. For histological analysis, Mann-Whitney and Kruskal-Wallis tests were applied for non-parametric comparison of scores between each group. The scores ranged from 1 to 4, with 1 representing no tissue injury and 4 representing severe damage. Hemodynamic, echocardiographic, and biochemical analyses were performed by ANOVA with Dunnet's post-hoc analysis. Statistical significance for the oxylipin analyses was calculated by one-way ANOVA followed by a Tukey post-hoc test. Results with $p<0.05$ were considered significant. The statistical software packages SPSS 15.0, SPSS version 24, and Graphpad Prism 5 were utilized to perform the statistical analyses

Chapter 4: Results

4.1 Murine Echocardiography

Baseline echocardiographic parameters including HR, IVS, PWT, LVEDD, and LVEF were similar between all treatment groups (Table 2). HR and PWT remained within the normal physiological range for all treatment groups for the 6-week study duration.

In mice treated with RC+DOX+TRZ, left ventricular end diastolic diameter (LVEDD) increased from 2.8±0.2 mm at baseline to 4.3±0.2 mm by week 6. Prophylactic administration of either PER or FLX alone partially prevented adverse LV remodelling with LVEDD values of 3.4±0.3 mm and 3.5±0.2 mm, respectively ($P<0.05$) (Figure 6). Interestingly, concomitant administration of PER+FLX did not provide synergistic cardioprotection at attenuating increased LVEDD in mice treated with DOX+TRZ.

Additionally, the left ventricular ejection fraction (LVEF) in mice treated with RC+DOX+TRZ decreased from 75±2% at baseline to 37±3% at week 6. Prophylactic treatment with either PER or FLX alone partially attenuated LV systolic dysfunction with LVEF values of 63±2% and 62±2%, respectively ($P<0.05$) (Figure 7). Prophylactic treatment with the combination of PER+FLX, however, was not synergistic at preventing LV systolic dysfunction.

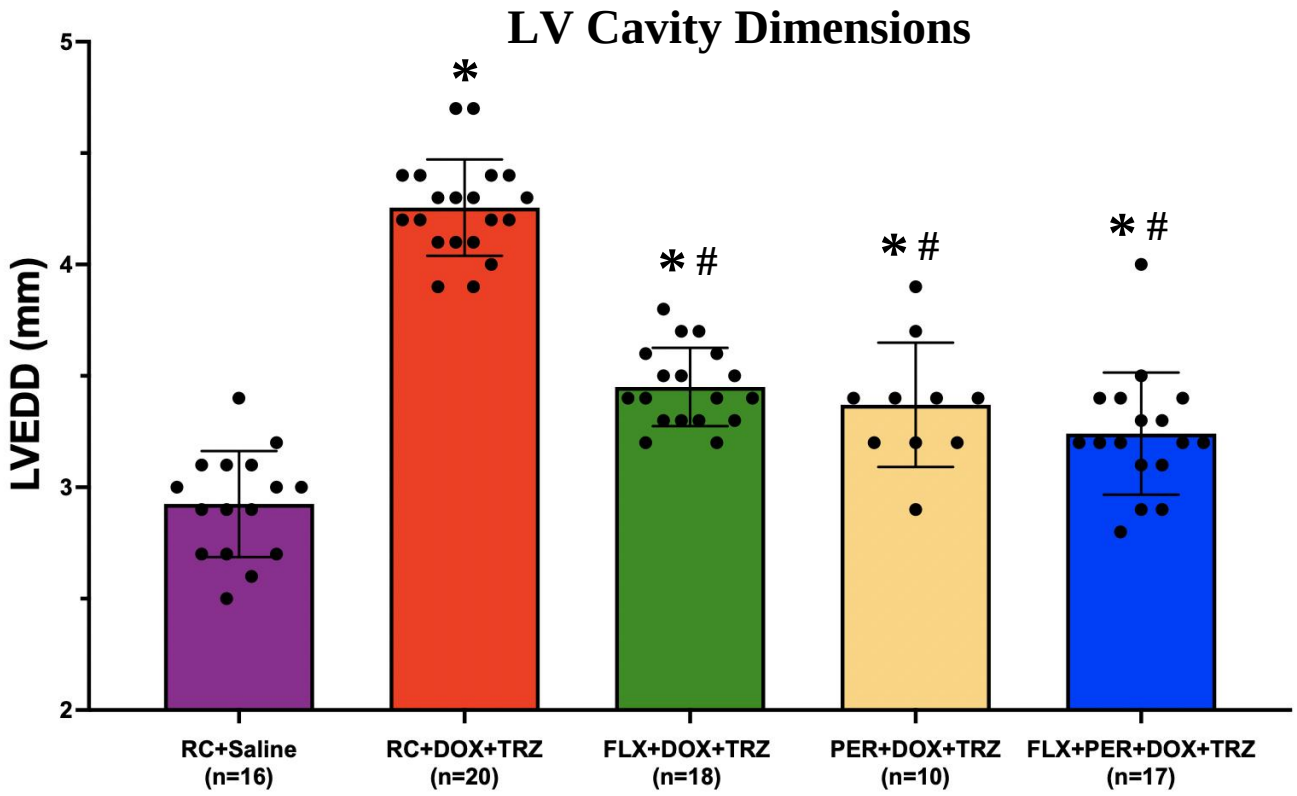
Table 2. Echocardiographic parameters at 6 weeks in C57Bl/6 mice receiving Saline or DOX+TRZ with daily prophylactic treatment of either FLX, PER, or FLX+PER.

Variable	Group	Baseline	Week 6	p value
HR (beats per minute)	RC+Saline (n=16)	694±6	690±7	0.84
	RC+DOX+TRZ (n=20)	687±9	693±6	0.81
	FLX+DOX+TRZ (n=18)	693±5	690±4	0.82
	PER+DOX+TRZ (n=10)	688±7	692±5	0.71
	FLX+PER+DOX+TRZ (n=17)	691±4	689±3	0.82
PWT (mm)	RC+Saline (n=16)	0.81±0.01	0.81±0.02	0.99
	RC+DOX+TRZ (n=20)	0.82±0.02	0.82±0.01	0.98
	FLX+DOX+TRZ (n=18)	0.82±0.01	0.81±0.02	0.92
	PER+DOX+TRZ (n=10)	0.82±0.02	0.82±0.01	0.98
	FLX+PER+DOX+TRZ (n=17)	0.82±0.01	0.82±0.02	0.97
LVEDD (mm)	RC+Saline (n=16)	2.8±0.1	2.9±0.1	0.78
	RC+DOX+TRZ (n=20)	2.8±0.1	4.5±0.2*	<0.05
	FLX+DOX+TRZ (n=18)	2.8±0.2	3.6±0.2$^{*\#}$	<0.05
	PER+DOX+TRZ (n=10)	2.8±0.1	3.5±0.2$^{*\#}$	<0.05
	FLX+PER+DOX+TRZ (n=17)	2.8±0.2	3.4±0.3$^{*\#}$	<0.05
LVEF (%)	RC+Saline (n=16)	74±2	74±3	0.92
	RC+DOX+TRZ (n=20)	75±2	34±2*	<0.05
	FLX+DOX+TRZ (n=18)	73±4	61±2$^{*\#}$	<0.05
	PER+DOX+TRZ (n=10)	74±3	62±2$^{*\#}$	<0.05
	FLX+PER+DOX+TRZ (n=17)	74±3	64±2$^{*\#}$	<0.05

DOX, Doxorubicin; FLX, Flaxseed; HR, Heart Rate; LVEDD, Left Ventricular End-Diastolic Diameter; LVEF, Left Ventricular Ejection Fraction; PER, Perindopril; PWT, Posterior Wall Thickeness; RC, Regular Chow; SD, Standard Deviation; TRZ, Trastuzumab.

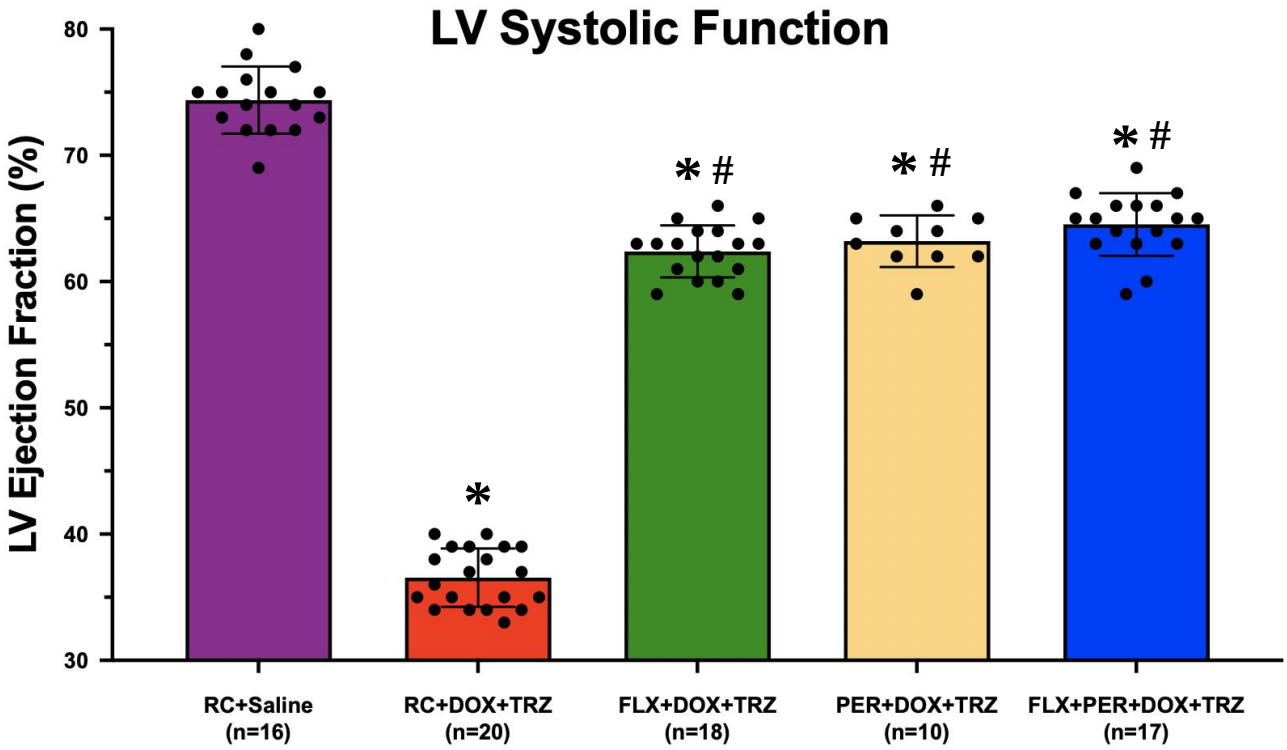
The values are presented as mean ± SD. *$p<0.05$ RC+DOX+TRZ vs. RC+Saline. $^{*\#}p<0.05$ FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. RC+DOX+TRZ & RC+Saline.

Figure 6. Changes in LVEDD of mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ.



C57Bl/6 female mice treated with RC+DOX+TRZ demonstrated LV cavity dilatation as show by increased LVEDD values at week 6. Treatment with FLX, PER, or FLX+PER significantly attenuated the LVEDD cavity dilation associated with DOX+TRZ administration. Data are expressed as mean±SD. *p<0.05 RC+DOX+TRZ vs. RC+Saline. $^{*\#}$p<0.05 FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. RC+DOX+TRZ & RC+Saline. DOX, Doxorubicin; FLX, Flaxseed; LV, Left Ventricle; LVEDD, Left Ventricular End Diastolic Diameter; PER, Perindopril; RC, Regular Chow; SD, Standard Deviation; TRZ, Trastuzmab

Figure 7. Changes in LVEF of mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ.

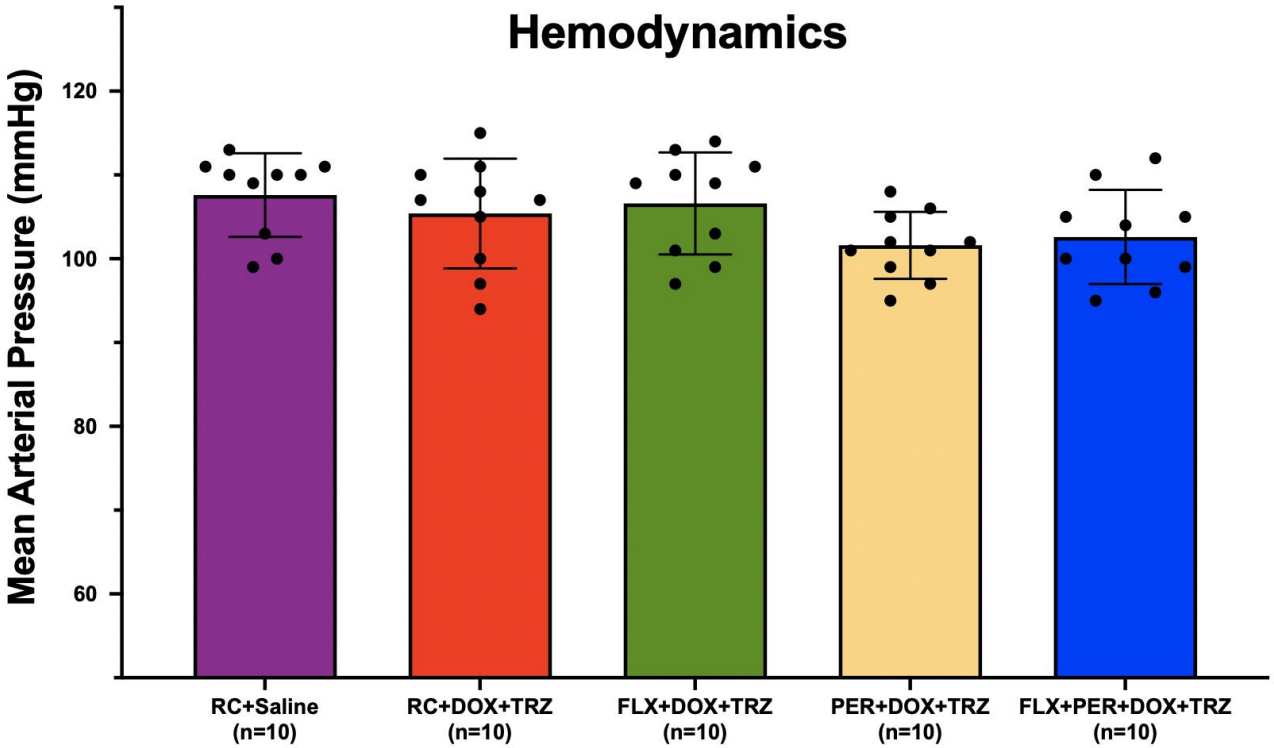


C57Bl/6 female mice treated with RC+DOX+TRZ demonstrated significantly impaired LV systolic function as demonstrated by LVEF values at week 6. Treatment with FLX, PER, or FLX+PER significantly improved the LVEF in animals administered DOX+TRZ. Data are expressed as mean±SD. *$p<0.05$ RC+DOX+TRZ vs. RC+Saline. $^{*\#}p<0.05$ FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. RC+DOX+TRZ & RC+Saline. DOX, Doxorubicin; FLX, Flaxseed; LV, Left Ventricle; PER, Perindopril; RC, Regular Chow; SD, Standard Deviation; TRZ, Trastuzmab

4.2 Hemodynamics

There was no statistically significant differences in MAP at week 6 compared to baseline in all study animals (p=NS). Additionally, prophylactic administration with FLX, PER, or FLX+PER did not significantly alter MAP at week 6 (Figure 8).

Figure 8. Changes in MAP of mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ.



C57Bl/6 female mice treated with DOX+TRZ did not show any significant alterations in MAP when compared to RC+Saline control. Additionally, prophylactic administration with FLX, PER, or FLX+PER did not result in any observed changes in MAP. Data are expressed as mean±SD. DOX, Doxorubicin; FLX, Flaxseed; MAP, Mean Arterial Pressure; PER, Perindopril; RC, Regular Chow; SD, Standard Deviation; TRZ, Trastuzmab

4.3 Histologic analysis

Mice treated with RC+Saline demonstrated normal cardiomyocyte integrity. Treatment with RC+DOX+TRZ, resulted in significant myofibril degradation, vaculozation and loss of sarcomere integrity. Prophylactic administration of FLX, PER, and FLX+PER partially prevented the adverse histopathological consequences of DOX+TRZ treatment (Figure 9).

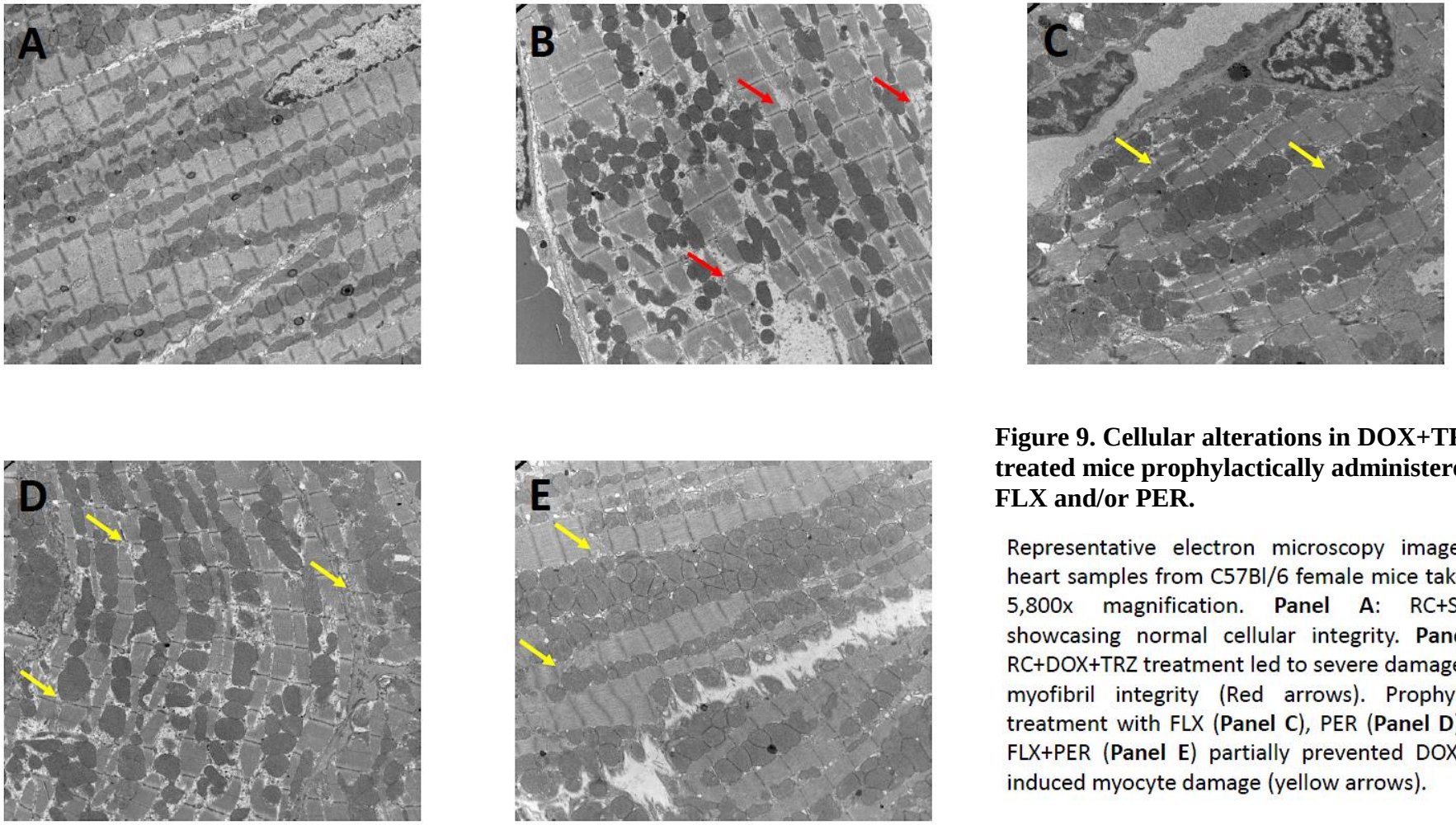


Figure 9. Cellular alterations in DOX+TRZ-treated mice prophylactically administered FLX and/or PER.

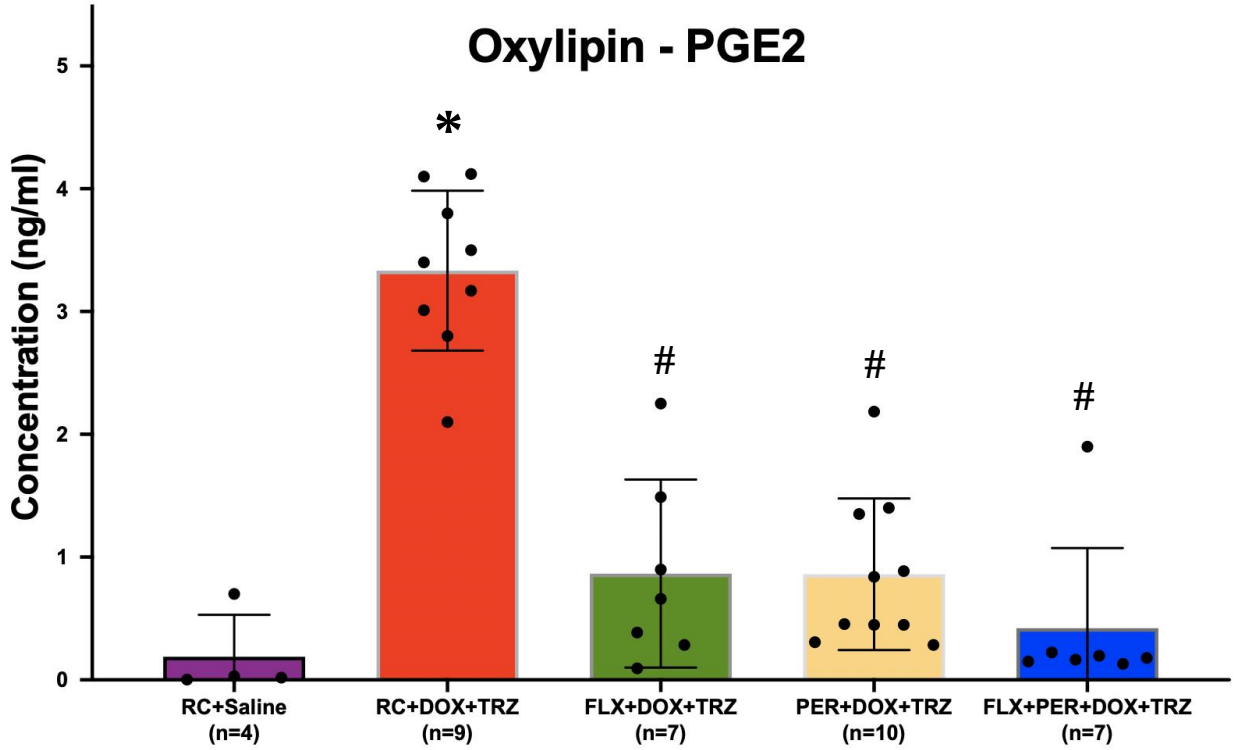
Representative electron microscopy images of heart samples from C57Bl/6 female mice taken at 5,800x magnification. **Panel A**: RC+Saline showcasing normal cellular integrity. **Panel B**: RC+DOX+TRZ treatment led to severe damage and myofibril integrity (Red arrows). Prophylactic treatment with FLX (**Panel C**), PER (**Panel D**) and FLX+PER (**Panel E**) partially prevented DOX+TRZ induced myocyte damage (yellow arrows).

4.4 Oxylipin Analysis

There was a significant increase in the the concentration of inflammatory oxylipins prostaglandin D2 (PGD2) and prostaglandin E2 (PGE2) in mice treated with RC+DOX+TRZ ($p<0.05$) (Figure 10 & 11). Prophylactic administration with either FLX or PER attenuated elevations in this inflammatory oxylipin ($p<0.05$). Interestingly, the combined effect of FLX+PER was not synergistic at reducing concentrations of this inflammatory oxylipin.

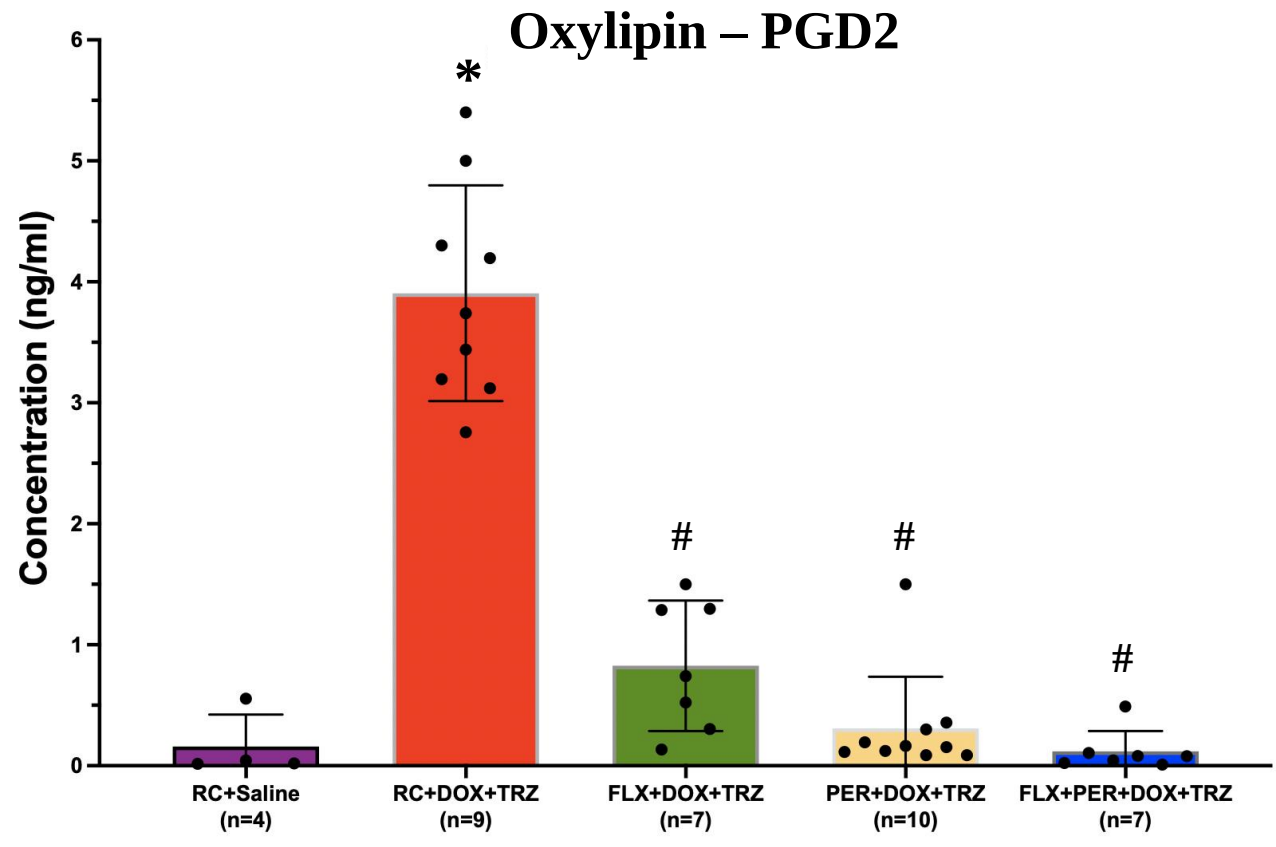
Additionally, mice treated with RC+DOX+TRZ displayed a significant increase in the concentration of 9-hydoxyoctadecadienoic acid (9-HODE) associated with oxidative stress and immune cell infiltration ($p<0.05$). Pre-treatment with FLX, PER, or FLX+PER was able to attenuate this oxidative stress biomarker ($p<0.05$).

Figure 10. Changes in PGE2 concentration in mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ.



C57Bl/6 female mice treated with RC+DOX+TRZ demonstrated a significant increase in the inflammatory oxylipin PGE2 at week 6. Treatment with FLX, PER, or FLX+PER significantly attenuated this increase in animals administered DOX+TRZ. Data are expressed as mean±SD. *$p<0.05$ RC+DOX+TRZ vs. RC+Saline. $^{\#}p<0.05$ FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. RC+DOX+TRZ. DOX, Doxorubicin; FLX, Flaxseed; PER Perinodpril; PGE2, Prostaglandin E2; RC, Regular Chow; SD, Standard Deviation; TRZ, Trastuzmab

Figure 11. Changes in PGD2 concentration in mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ.



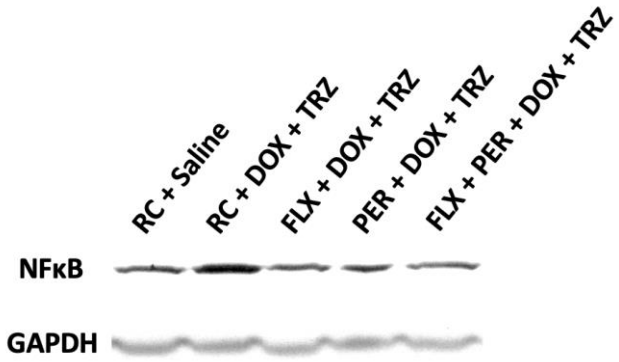
C57Bl/6 female mice treated with RC+DOX+TRZ demonstrated a significant increase in the inflammatory oxylipin PGD2 at week 6. Treatment with FLX, PER, or FLX+PER significantly attenuated this increase in animals administered DOX+TRZ. Data are expressed as mean±SD. *$p<0.05$ RC+DOX+TRZ vs. RC+Saline. $^{\#}p<0.05$ FLX+DOX+TRZ or PER+DOX+TRZ or PER+FLX+DOX+TRZ vs. RC+DOX+TRZ. DOX, Doxorubicin; FLX, Flaxseed; PER Perinodpril; PGE2, Prostaglandin E2; RC, Regular Chow; SD, Standard Deviation; TRZ, Trastuzmab

4.5 Western Blotting

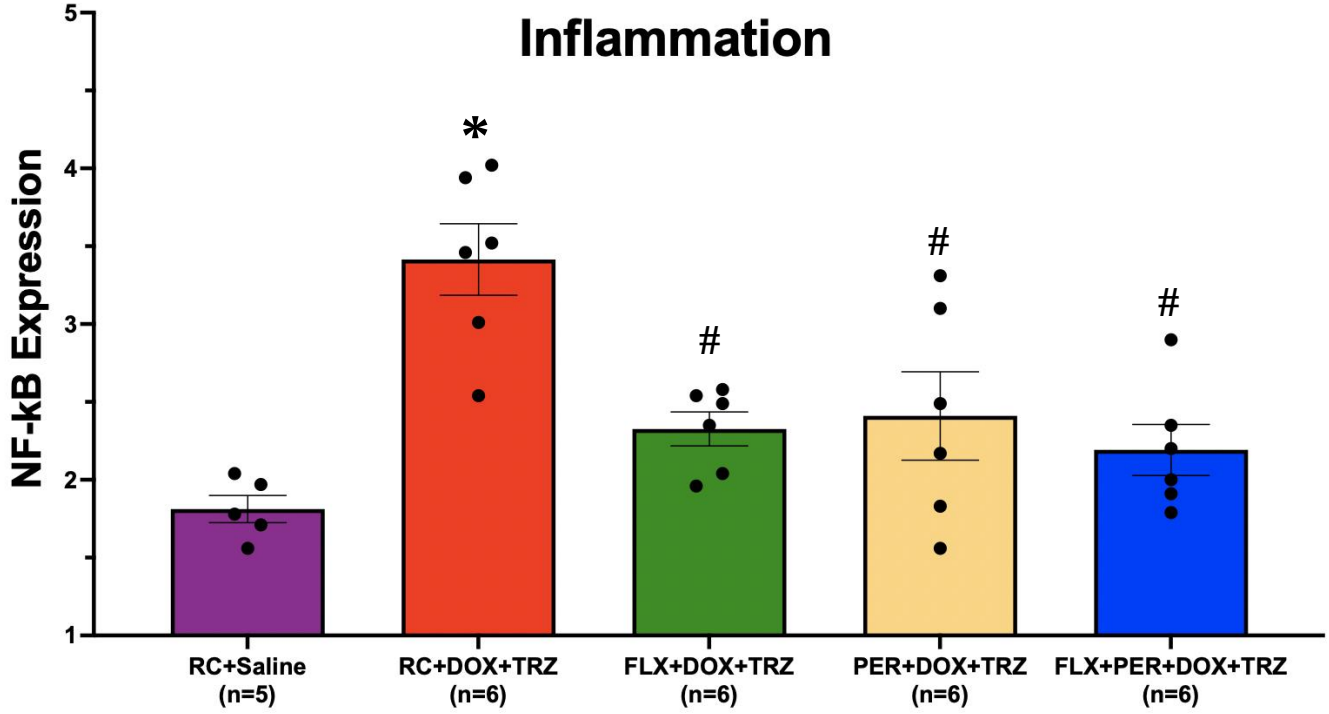
Mice treated with RC+DOX+TRZ, demonstrated a 2-fold increase in NF-κβ expression when compared to RC+Saline treated mice at week 6 ($p<0.05$). Prophylactic administration with either FLX or PER attenuated this increase with a 1.3 and 1.2-fold increase in NF-κβ expression at week 6, respectively ($p<0.05$). Interestingly, the combination of FLX+PER did not provide synergistic cardioprotection with a 1.2-fold increase in NF-κβ expression experienced at week 6 (Figure 12).

Figure 12. Changes in NF-κβ expression in mice prophylactically treated with FLX, PER, or FLX+PER receiving DOX+TRZ.

A.



B.



A. Representative western blot. **B.** Changes in NF-κβ expression of mice prophylactically administered FLX, PER, or FLX+PER treated with DOX+TRZ. *p<0.05 RC+DOX+TRZ vs. RC+Saline. #p<0.05 FLX+DOX+TRZ or PER+DOX+TRZ, or FLX+PER+DOX+TRZ vs. RC+DOX+TRZ and RC+Saline. DOX, doxorubicin; FLX, flaxseed; PER, perindopril; RC, regular chow; TRZ, trastuzumab.

Chapter 5: Discussion

5.1 Overall Summary

Breast cancer remains the most common cancer among Canadian women.[1] Marked advances in the prevention, detection, and management of breast cancer have resulted in reduced morbidity and mortality; however, long-term cardiovascular side effects remain a serious concern.[1,11,13,14,18] Cardio-Oncology is a collaborative field responsible for the evaluation and management of cardiovascular complications in cancer patients. The lack of adequate remedial treatment options for patients who develop chemotherapy-induced cardiotoxicity has urged the discovery of novel preventative strategies. Previous studies have investigated the cardioprotective role of pharmaceuticals including aliskiren, valsartan, and PER, as well as nutraceuticals including FLX in this setting.[66,102] However, to date, no studies have investigated the equivalence and combined effects of both a nutraceutical and pharmaceutical in the prevention of chemotherapy-induced cardiotoxicity in a chronic *in vivo* female murine model.

Our study demonstrated that prophylactic administration with either FLX or PER: i) attenuated adverse LV cavity remodelling; ii) ameliorated myofibrillar disarray; iii) reduced inflammatory oxylipins; and iv) lowered concentrations of the inflammatory biomarker NF-κβ in a chronic *in vivo* model of DOX+TRZ. However, the combined effects of FLX+PER did not exhibit synergistic cardioprotection.

5.2 Cardiovascular remodeling

Originally used for the preoperative assessment of mitral stenosis, the utility of echocardiography was first discovered in 1953.[136] This non-invasive imaging modality has since become routinely used in most cardiac examinations. Additionally, transthoracic echocardiography has become the gold-standard for the serial monitoring of cardiac functioning for patients before, during, or after chemotherapy due to its availability, reproducibility, and versatility.[14] As such, conventional echocardiographic indexes are frequently evaluated in both preclinical and clinical Cardio-Oncology research investigations.

In the Cardio-Oncology setting, it is well established that the combinational treatment with DOX+TRZ leads to adverse cardiovascular remodelling in pre-clinical models. In a 2019 study, Rodrigues *et al*. (2019) investigated the early myocardial changes in a chronic rabbit model of DOX-induced cardiotoxicity.[137] During the course of the 8-week study, echocardiographic evaluation revealed LV cavity dilatation and hypertrophy in rabbits treated with 1mg/kg DOX intraveneously administered twice weekly for a total of 8-weeks.[137] Interetingly, throughout the course of the study LVEF was preserved.[137] Similarly, a previous study by Akolkar *et al*. (2015), investigated the benefits of prophylactic RAS antagonists administration in the prevention of chemotherapy-induced cardiotoxicity.[66] Specifically, mice were randomized to receive prophylactic treatment with oral PER, aliskiren, or valsartan, for a total of 13 weeks. Mice were further randomized to receive an i.p. injection of DOX, TRZ, or the combination of DOX+TRZ weekly for a total of 5 weeks. Through serial echocardiography, they demonstrated a significant increase in LV cavity dimensions associated with DOX+TRZ treatment. Specifically, mice treated with DOX+TRZ demonstrated an increase in LVEDD from 3.1±0.2mm at baseline to 4.6±0.3mm

at week 13 ($p<0.05$). Pretreatment with PER, aliskiren, and valsartan attenuated LV cavity dilitation from 4.5±0.2mm to 3.9±0.2mm, 3.6±0.2mm, and 4.0±0.2mm, respectively, at week 13 ($p<0.05$). These results are congruent with the current study whereby treatment with DOX+TRZ resulted in a significant increase in LVEDD from 2.8±0.2mm at baseline to 4.3±0.2mm at study endpoint. Prophylactic administration with either FLX or PER significantly attenuated this LV cavity dilatation with an increase in LVEDD from 2.8±0.2mm at baseline only to 3.5±0.2 and 3.4±0.3mm at week 6, respectively ($p<0.05$). Most interestingly, however, the combination of FLX+PER did not appear to synergistically attenuate LV cavity dilatation due to DOX+TRZ.

In a more recent study, Asselin *et al*. (2020) investigated the cardioprotective effects of dietary FLX and its bioactive components (ALA and SDG) in a chronic *in vivo* murine model of DOX+TRZ-mediated cardiotoxicity.[102] Throughout this 6-week study, mice were randomized to receive RC, FLX, ALA, or SDG-supplemented diets for a 3-week period. Following the dietary run-in period, mice were further randomized to receive a three weekly i.p. injection with DOX, TRZ, or DOX+TRZ. Serial echocardiography revealed a significant decrease in LVEF associated with DOX+TRZ administration.[102] Specifically, mice treated with DOX+TRZ demonstrated a decrease in LVEF from 75±2% at baseline to 37±3 at week 6 ($p<0.05$). Treating the animals prophylactically with FLX, ALA, and SDG was partially cardioprotective with LVEF values of 62±2%, 61±3%, and 62±4%, respectively, at week 6 ($p<0.05$). These results are validated by our current 6-week study, whereby treatment with FLX and PER attenuated LVEF declines from 74% to 61±2% and 62±2%, respectively, at week 6 ($p<0.05$). Importantly, however, the current study confirmed that the combination of FLX+PER, when administered prophylactically, does not

synergistically preserve systolic dysfunction with a drop in LVEF from 74±3% at baseline to 64±2% at week 6.

Finally, in a rat model of DOX-induced cardiomyopathy, Lodi *et al*. (2019) compared the cardioprotective effects of a prophylactic HF treatment relative to a conventionally scheduled treatment commenced at a later stage.[138] Specifically, they compared whether treatment with oral PER (2mg/kg), bisoprolol (2.5mg/kg), or eplerenone (6.25mg/kg) one week before DOX administration would be superior to initiating HF medications one-month following DOX treatment. [138] Rats were randomized to receive one of four treatments including: (i) Saline; (ii) DOX (6-cycles of 1.5mg/kg intravenously); (iii) DOX prophylactically treated with bisoprolol, PER, and eplerenone; or (iv) DOX conventionally treated with bisoprolol, PER, and eplernone. Echocardiographic parameters were assessed in sedated animals at baseline and at follow-up days 51 and 80. Overall, it was found that systolic function declined significantly in the rats treated with DOX as compared to control ($p<0.05$). Interestingly, LVEF decreased from 83% to 72% in the rats treated with HF medications using a conventional schedule, whereas LVEF only reduced from 84% to 81% in rats treated prophylactically with bisoprolol, PER, or eplerenone ($p<0.05$).[138] This study elegantly illustrates the advantage of prophylactic cardioprotection compared to late-applied treatments in the setting of DOX-induced cardiotoxicity. This is corroborated with our study whereby the prophylactic administration of FLX, PER, or FLX+PER effectively blocked adverse cardiovascular remodeling resulting from DOX+TRZ treatment. This underscores the importance of using preventative measures, such as in the current study, in the setting of chemotherapy-induced cardiotoxicity.

5.3 Hemodynamics

Following the administration of DOX+TRZ, a number of hemodynamic alterations including heart rate and blood pressure have been studied, with conflicting results. Sharma *et al*. (2011) investigated the cardioprotective role of rosuvastatin in an acute *in vivo* model of DOX-induced cardiomyopathy in rats.[139] Using a non-invasive tail cuff method, they demonstrated that rats treated with a single i.p injection of 30mg/kg DOX had a significant increase in systolic, diastolic, and mean BP by 50%, 57%, and 55%, respectively.[139] Additionally, rats treated with 30mg/kg DOX experienced a significant increase in HR, mean tail blood flow and mean tail blood volume as compared to controls.[139] In contrast, Razmaraii *et al*. (2020) investigated the hemodynamic and echocardiographic alterations associated with chronic DOX administration in rats.[140] Overall, they demonstrated HR significantly decreased in rats subject to a cumulative dose of 12mg/kg or 15mg/kg DOX administered in six equal doses over a two week period. In their study, HR was measured in rats treated with saline, DOX (12mg/kg, i.p.), or DOX (15mg/kg, i.p.). Notably, treatment with DOX (12mg/kg, i.p.), or DOX (15mg/kg, i.p.) resulted in a 17% and 20% reduction in HR as compared to controls.[140] Furthermore, Lodi *et al*. (2019) found that treatment with DOX significantly increased the HR of animals compared to controls.[138] Specifically, while saline treated rats exhibited a HR of 406±11bpm, animals treated with DOX demonstrated an significant increase in HR to 464±19bpm ($p=0.0193$). Finally, Baniahmad *et al*. (2020) investigated the cardioprotective effects of vanillic acid against DOX-induced cardiotoxicity in rats.[141] Overall, the administration of 6 doses of DOX (2.5mg/kg i.p.) three times per week for two weeks resulted in a significant reduction in HR and SBP. Specifically, when compared to control, DOX administration decreased HR and SBP by 19% and 28%, respectively ($p<0.001$).[141] Pretreatment with either 20mg/kg or 40mg/kg vanillic acid was able to restore HR and SBP to near normal

levels.[141] Interestingly, in the same study, pretreatment with dexrazoxane at 50mg/kg, 30 minutes before DOX administration did not protect against DOX-induced hemodynamic changes.[141] Overall, all of the aforementioned pre-clinical studies demonstrate that DOX+TRZ has varying effects on HR and BP, dependent on the duration of treatment with DOX+TRZ. As compared to these previous studies, in our current chronic 6 week *in vivo* model of DOX+TRZ-mediated cardiotoxicity, no hemodynamic differences in HR nor BP were observed when compared to controls. One could speculate that the lack of hemodynamic alterations seen was due to our abbreviated study timeline, that may not have allowed sufficient time for hemodynamic manifestations of DOX+TRZ treatment.

5.4 Cardiomyocyte injury and mitochondrial disarray

Loss of cardiomyocyte integrity is a hallmark pathology associated with DOX treatment.[66,77,102] A number of basic science studies have investigated the histopathological aspects of DOX treatment.[49,66,77,102,115] Mitochondrial swelling, vacuolization of the cytoplasm, dilatation of the sarcotubular system, and formation of lysosomal bodies are well established histopathologic manifestations of 15mg/kg DOX in neonatal rats treated with over a 2-week period.[66,102,142] Similarly, Rea *et al*. (2016) sought to ascertain the cardiac ultrastructural manifestation of DOX-induced cardiotoxicity in a murine model.[143] Specifcally, mice were randomized to receive an i.p. injection with either 0.9% saline or DOX (2.17mg/kg) daily for a total of 7 days.[143] Histological analysis revealed that DOX treatment increased fibrosis, cardiomyocyte diameter, and cardiomyocyte apoptosis.[143] Finally, Argun *et al*. (2016) sought to investigate the cardioprotective effects of metformin against DOX-induced cardiotoxicity in wistar albino rats.[144] In their study, rats were randomized to receive an i.p. injection with either saline, or DOX (4mg/kg) twice a week,

for a total cumulative dose of 16mg/kg. Upon histological analysis, they found that DOX treatment resulted in disordered myocardial muscle fibers, loss of myofibril assembly, and intracytoplasmic vacuole formation.[144] Interestingly, concurrent treatment with metformin ameliorated the pathologic manifestations due to DOX.

Similarly, treatment with TRZ has been shown to induce histopathological manifestation similar to that of DOX.[66,102] In a recent study, Laird-Fick *et al.* (2020) sought to investigate the early morphological changes in cardiac ultrastructure after subcutaneous administration of TRZ in rabbits.[56] Animals were subject to a loading dose of 8mg/kg, followed by 3 weekly subcutaneous injections with 6mg/kg TRZ. Overall, this study found that 4 doses of TRZ (cumulative dose of 26mg/kg) resulted in interstitial and perivascular infiltration of lymphocytes and macrophages, rare apoptosis, and myofiber necrosis.[56] Additionally, as demonstrated by transmission electron microscopy, TRZ treatment was also shown to decrease the number of functional mitochondria per field when compared to control. Finally, TRZ-treated rabbits demonstrated the largest mean volume per mitochondria compared to control-treated animals.[56]

The histologic manifestations resulting from simultaneous treatment with DOX+TRZ has been demonstrated in preclinical investigations. In a study by Kertmen *et al*., (2015) they investigated the timing sequence of DOX and TRZ administration that results in the most severe cardiomyocyte ultrastructural changes. Specifically, mice were randomized to one of four drug regimens including: (i) 0.9% Saline; (ii) DOX (5mg/kg) on day 1, followed by TRZ (10mg/kg) on day 15; (iii) TRZ (10mg/kg) on day 1, followed by DOX (5mg/kg) on day 15; or (iv) DOX (5mg/kg) and TRZ (10mg/kg) simultaneously administered on day 1. Through transmission electron

microscopy, they demonstrated that TRZ therapy followed by DOX resulted in the most severe ultrastructural changes including prominent perivascular dilations and separations between the myofibrils in large areas. In contrast, concurrent treatment with both DOX+TRZ resulted in a mild degree of dilation in the perinuclear cristernae and loss of myobril integrity in focal areas. Our lab has corroborated these prior studies whereby treatment with DOX+TRZ resulted in significant ultrastructural changes including a reduction in a significant loss of myofibril assembly and cytoplasmic vacuoliazation in the current study. Remarkably, the prophylactic administration of either PER or FLX alone reduced myofibril degradation, intracellular vacuolization and loss of sarcomere integrity upon histological analysis in mice treated with DOX+TRZ.[66,102] The results from our current study verify these findings through which treatment with FLX and PER alleviated the ultrastructural changes resulting from DOX+TRZ treatment. However, for the first time, we have demonstrated that the concomitant administration with FLX+PER was not synergistic at attenuating the ultrastructural cardiomyocyte changes exhibited by DOX+TRZ.

5.5 Inflammation

Several physiological mechanisms have been implicated in the development of DOX+TRZ-mediated cardiotoxicity. Among these mechanisms, there has been an increased awareness on the role of OS and inflammation leading to apoptosis, and myocardial fibrosis, and heart failure.[66,77,78,80] Specifically, DOX+TRZ treatment is associated with a substantial rise in ROS leading to DNA damange, lipid-peroxidation, and inflammation. As such, molecular biomarkers including oxylipin concentrations and NF-kB expression are a reliable and effective measure of cellular stress and inflammation.

Oxylipins are a group of bioactive metabolites endogenously produced through the oxygenation of polyunsaturated fatty acids.[145] Specifically, these bioactive metabolites are formed via the cyclooxygenase (COX), lipoxygenase, and cytochrome P450 pathways resulting in a diverse range of prostaglandins, thromboxanes, and mono-, di-, and tri-hydroxy fatty acid metabolites.[145,146] Oxylipins have been implicated in a range of physiological and pathophysiological processes including: (i) inflammation; (ii) immunity; (iii) cardiac function; and (iv) regulation of vascular tone.[145] Produced from arachidonic acid, eicosanoids have been involved in cardiovascular pathologies including atherosclerosis, vascular constriction, cardiac injury and dysfunction.[145] Similarly, COX-produced oxylipins including 6-Keto-PGF1α, PGF2α, PGE2, and PGD2 have been shown to be key regulators for physiologic and pathologic inflammatory responses.[145,147,148] Previous studies performed by our lab have shown a significant rise in the concentration of these COX-derived oxylipins in a chronic *in vivo* model of DOX+TRZ-mediated cardiotoxicity. Elevations in COX-derived oxylipins is congruent with prior studies indicating systemic inflammation as a key contributor to the pathogenesis of DOX+TRZ-mediated cardiotoxicity.[16,66,102] Fortunately, the prophylactic administration of several cardioprotective agents including pharmaceuctical agents such as RAS antagonsts and neutraceutical agents such as flaxseed are able to attenuate elevations in inflammatory oxylipins associated with chronic DOX+TRZ administration.[66,102] These results were corroborated in our current study, whereby pretreatment with FLX was able to attenuate elevations in inflammatory oxylipins PGD2 and PGE2 by 76% ($p<0.05$). Similarly, the prophylactic administration of PER attenuated elevations in PGD2 and PGE2 by 96% and 75%, respectively ($p<0.05$). However, the combined administration with both FLX+PER did not exhibit additive effects at attenuating elevations in these inflammatory oxylipins.

In addition to oxylipins, a central component of DOX+TRZ-mediated cardiotoxicity involves the up-regulation of pro-inflammatory mediators including NF-κβ. Once activated, NF-κβ serves to induce the production of several downstream pro-inflammatory biomarkers including interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor alpha (TNF-α).[16,102] Cardiac manifestations resulting form elevations in these inflammatory biomarkers include cardiac fibrosis and HF.[66,77,78,80] Numerous preclinical studies have investigated the impact of inflammation on both acute and chronic models of chemotherapy-induced cardiotoxicity.[16,66,77,78,102,141] In a recent preclinical study, Younis *et al*. (2020) investigated the cardioprotective capabilities of sandalwood oil in ameliorating DOX-induced cardiac abnormalities in rats.[149] In their rat model of DOX-induced cardiotoxicity, supplementation with sandalwood oil effectively abated the robust inflammatory response induced by DOX. The effectiveness of sandalwood oil at suppressing inflammation was shown by the attenuation of several pro-inflammatory mediators including IL-1β, TNF-α, and NF-κβ.[149] This study has served to corroborate prior studies performed by our lab whereby elevations in inflammatory biomarkers is a key contributor to the pathogenesis of DOX+TRZ-mediated cardiotoxicity. Specifically, our lab has shown that several markers of inflammation including NF-κβ, TNF-α, IL-1β, and IL-6 are elevated in a chronic 6-week study of DOX+TRZ-mediated cardiotoxicity in a female murine model.[66,77,78,80,102] Interestingly, these biomarkers were significantly attenuated in animals pretreated with the nutraceutical agent FLX. The results from our current study corroborate the aforementioned investigation whereby pretreatment with either PER or FLX alone was able to reduce elevations in NF-κβ concentrations observed in DOX+TRZ-treated mice by 31.4%, and 34.3%, respectively. Of interest, animals pretreated with concurrent use of both FLX+PER did not experience synergistic attenuation of inflammatory biomarkers in DOX+TRZ-treated mice. It can

be speculated that the lack of synergy exhibited is due to the similar cardioprotective mechanism demonstrated by both PER and FLX independently including attenuation of the NF-kB inflammatory pathway.[66,77,102]

Clinically, the role of inflammatory biomarkers among breast cancer patients receiving cardiotoxic chemotherapy is emerging.[150] In the placebo-controled, double-blind prevention of cardiac dysfunction during adjuvant breast cancer therapy (PRADA) study, circulating cardiovascular biomarkers were monitored in 121 women were receiving anthracycline-based chemotherapy.[151] Overall, it was found that contemporary doses of anthracyclines in breast cancer patients is associated with increased cardiac injury, fibrotic, and inflammatory biomarkers. Specifically, it was found that concentrations of C-reactive protein (CRP) and galectin-3 are elevated and contribute to both systemic inflammation and fibrosis.[150] Lastly, Todorova *et al.* (2020) determined that serum biomarkers of inflammation, hypercoagulability, and endothelial injury predicted subclinical doxorubicin-induced cardiotoxicity in breast cancer patients.[152] They found that elevations in the inflammatory indicator c-reactive protein (CRP) was able to detect subclinical doxorubicin-induced cardiotoxicity among breast cancer patients.[152] These exciting results increase the evidence surrounding the use of inflammatory biomarker surveillance to detect cardiotoxicity resulting from chemotherapy use.

5.7 Limitations

There are a number of limitations associated with the current study. First, we only evaluated the cardioprotective potential of FLX, PER, and FLX+PER in DOX+TRZ-mediated cardiotoxicity in a chronic *in vivo* female murine model. As breast cancer and the associated use of anticancer

therapies is not exclusive to females, further studies are warrented to evaluate the cardioprotective potential in male models. Second, while the current study administered DOX+TRZ concurrently, their use in the clinical setting is most commonly administered sequentially. To better recapitulate the clinical use of DOX+TRZ, further studies are warrented to characterize the cardiotoxicity and possible cardioprotection in a model whereby DOX and TRZ are administered sequentially. Third, the current study did not evaluate the impact of FLX or PER on the anti-neoplastic properties of DOX+TRZ. Further studies are warranted to ensure that FLX+PER consumption does not render DOX+TRZ ineffective.

5.8 Future Directions and Clinical Implications

The following investigations are warranted to fully characterize the potential clinical cardioprotective utility of FLX and PER in preventing DOX+TRZ-mediated cardiotoxicity. Specifically, preclinical and clinical investigations are warrented to determine the cardioprotective effects of FLX and PER in the *treatment* of DOX+TRZ-mediated cardiotoxicity. Second, future *in vitro* and *in vivo* studies are recommended to confirm that FLX and PER supplementation will not compromise the anti-neoplastic properties of DOX+TRZ. Finally, a multicenter, randomized-control trial investigating the effectiveness of FLX in preventing DOX+TRZ-mediated cardiotoxicity in breast cancer patients is warrented to fully characterize its clinical utility.

Clinically, the current study has tremendous implications. The current standard of practice in oncological patients is to cease vital anti-cancer therapies and intiate HF medications once overt cardiotoxicity has developed. In addition to providing a unique opportunity for their malignancy to spread, several of these cardioprotective agents are associated with adverse side effects

including lightheadness and fatigue.[66,102,153] While corroborating clinical trials are essential, our novel preclinical findings have shown that consumption of a nutraceutical agent, FLX, is comparable to the standard of care medication. As such, consumption of FLX may prove to be an favourable and feasibile alternative to PER due to its improved tolerability and many health benefits.[100,145]

Chapter 6: Conclusion

Our novel study has shown that the prophylactic administration of either FLX or PER was able to prevent the cardiotoxic effects of DOX+TRZ-mediated cardiotoxicity in a chronic *in vivo* female murine model. The prophylactic administration with the combination of FLX+PER, however, was not synergistic in attenuating the cardiotoxicity associated with DOX+TRZ.

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