The Cardioprotective Role of Flaxseed in the Prevention of Anthracycline and Trastuzumab Mediated Cardiotoxicity

by

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Abstract

Background: Cardio-Oncology focuses on the detection, prevention, and treatment of cardiovascular complications in individuals receiving cancer therapy. While the combination of Doxorubicin (DOX) and Trastuzumab (TRZ) reduces the progression and recurrence of breast cancer, these anti-cancer drugs are associated with significant cardiotoxic side effects. Little is known on the potential cardioprotective role of flaxseed (FLX) in the prevention of DOX+TRZ mediated cardiotoxicity.

Objective: To investigate whether prophylactic administration of FLX and its bioactive components, alpha-linolenic acid (ALA) or secoisolariciresinol-diglucoside (SDG), will be cardioprotective against DOX+TRZ mediated cardiotoxicity in a chronic *in vivo* female murine model.

Methods: A total of 195 wild-type C57Bl/6 female mice were divided into four groups and received daily prophylactic treatment with either: i) regular chow (RC); ii) FLX; iii) ALA; or iv) SDG for the entire 6-week study period. Within each arm, mice received 3 weekly injections of either: i) 0.9% Saline; ii) DOX (8 mg/kg); TRZ (3 mg/kg); or DOX+TRZ, at weeks 4, 5, and 6 to model chemotherapy associated cardiotoxicity. Following serial echocardiography, cardiac tissue was collected for histological analysis. Plasma oxylipins and biochemical analyses were performed to evaluate markers of: i) inflammation; ii) oxidative stress (OS) and apoptosis; and iii) mitochondrial dysfunction, using liquid chromatography electrospray ionization tandem mass spectrometry and Western blotting.

Results: In our chronic *in vivo* model of DOX and TRZ induced cardiotoxicity, prophylactic treatment with FLX, ALA, or SDG prevented the development of left ventricular systolic dysfunction (LVSD). The echocardiographic findings revealed that in RC+DOX treated mice, left ventricular ejection fraction (LVEF) decreased from 75±2% to 49±2% at week-6. Prophylactic administration of FLX, ALA, or SDG partially preserved LVEF with values of 66±3%, 63±3% and 65±4%, respectively. Similarly, in mice treated with RC+DOX+TRZ, the LVEF decreased from 73±2% to 38±2% at week-6. Addition of FLX, ALA, or SDG partially preserved LVEF with values of 61±2%, 60±3% and 61±4%, respectively. Animals treated with RC+DOX or RC+DOX+TRZ demonstrated increased loss of cellular integrity and myofibril disarray, which was partially attenuated by the FLX, ALA, and SDG diets. Plasma analysis confirmed that COX-derived oxylipins (inflammation) and 8,9-DiHETrE (OS) concentrations were significantly upregulated in the RC+DOX+TRZ treated mice. However, the FLX and ALA diets significantly downregulated COX-derived metabolites while 8,9-DiHETrE concentrations were significantly reduced by prophylactic treatment with FLX, ALA, and SDG. Western blot analysis detected a significant increase in the expression of NF-κB (inflammation), PARP (apoptosis), Bax/Bcl-xL (apoptosis), and Bnip3 (mitochondrial dysfunction) in the RC+DOX+TRZ group. However, these increases were attenuated by the prophylactic treatment with either FLX, ALA, or SDG.

Conclusion: In a chronic *in vivo* female murine model of DOX+TRZ mediated cardiotoxicity, FLX, ALA, and SDG partially prevented adverse cardiovascular remodeling by attenuating the degree of inflammation, apoptosis, and mitochondrial dysfunction.

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In loving memory of

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List of Abbreviations

A	Adriamycin (Doxorubicin)
AA	Arachidonic acid
AC	Adriamycin-Cyclophosphamide
ACE	Angiotensin converting enzyme
AC-T	Adriamycin:Cyclophosphamide:Paclitaxel
ALA	Alpha-linolenic acid
ANG-II	Angiotensin-II
ANOVA	Analysis of variance
AKT	Protein kinase B
ARB	Angiotensin receptor blocker
ARG	Amphiregulin
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine Triphosphate
AT_1	Angiotensin-1 receptor
AUC	Area under the curve
Bax	Bcl-2 associated X protein
β–Blocker	Beta blocker
BCIRG 006	Breast Cancer International Research Group 006 Data Trial
Bcl-2	B-cell lymphoma 2
Bcl-X_L	B-cell lymphoma extra-large
Bcl-X_S	B-cell lymphoma extra-small

Bnip3	Bcl-2 interacting protein 3
BP	Blood pressure
BRCA	Breast cancer gene
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
BSA	Bovine serum albumin
BTC	Betacellum
B1	Thiamine
B2	Riboflavin
B3	Niacin
B5	Pantothemic acid
C	Cyclophosphamide
CAD	Coronary artery disease
CAF/FAC	Cyclophosphamide:Doxorubicin:5-Fluorouricil
CCS	Canadian Cardiovascular Society
CDKI	Cyclin dependent kinase inhibitor
CF	Cholesterol:flaxseed
CH	Cholesterol
CH_3	Methyl functional group
CHF	Congestive heart failure
CISH	Chromogenic in situ hybridization
CLEOPATRA	Clinical evaluation of Pertuzumab and Trastuzumab

CMF	Cyclophosphamide:Methotrexate:5-Fluorouricil
CMR	Cardiac magnetic resonance imaging
c-neu	Human epidermal growth factor receptor 2
COOH	Carboxylic acid functional group
COX	Cyclooxygenase
CTRCD	Cancer therapeutics-related cardiac dysfunction
CVD	Cardiovascular disease
CXCL1	Chemokine C-X-C motif ligand 1
DAC	Docetaxel:Doxorubicin:Cyclophosphamide
DBP	Diastolic blood pressure
DCIS	Ductal carcinoma in situ
ddH_2O	Deionized water
DFS	Disease free survival
DHA	Docosahexenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
DOX	Doxorubicin
DRI	Direct renin inhibitor
E	Epirubicin
EA	Ellagic Acid
ECL	Enhanced chemiluminescence

EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
EGTA	Ethylene Glycol-bisβ-monoethyl ether tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ENU	Ethylnitrosourea
ENX	Enoxaparin
EPA	Eicosapentaenoic acid
ER	Estrogen receptor
ErbB	Epidermal growth factor receptor
ErbB2	Epidermal growth factor receptor 2
ERD	Estrogen receptor downregulator
ERG	Epiregulin
ESI	Electrospray ionization
ETC	Electron transport chain
Fab	Fragment antigen-binding region
FASG	French adjuvant study group
FDA	Food and drug association
FEC	5-Fluorouricil:Epirubicin:Cyclophosphamide
FEC	5-Fluorouricil:Epirubicin:Cyclophosphamide:Docetaxel
FID	Flame ionization detection

FISH	Fluorescence in situ hybridization
FLX	Flaxseed
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
GC-FID	Gas chromatography and flame ionization detection
GHS	Glutathione peroxidase
GPCR	G-protein coupled receptor
GSSG	Oxidized glutathione
HB-EGF	Heparin-binding EGF-like growth factor
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HER	Human epidermal growth factor receptor
HER1	Human epidermal growth factor receptor 1
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
HERA	Herceptin Adjuvant Trial
HF	Heart failure
HIF-1α	Hypoxia-inducible factors-1 alpha
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC	High performance liquid chromatography
HR	Heart rate

HRG	Hereguling
HTN	Hypertension
IHC	Immunohistochemistry
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
INF-α	Interferon-alpha
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
IR	Ischemia/reperfusion
i.v.	Intravenous
IVS	Interventricular septal thickness
JNK	c-Jun N-terminal kinase
LC	Liquid chromatography
LC3-II	Microtubule light chain 3
LDL	Low-density lipoprotein
$LDL^{(-/-)}$	Low-density lipoprotein receptor-deficient
LHRH	Luteinizing hormone releasing hormone
LOX	Lipoxygenase
LV	Left ventricle
LVEDD	Left ventricular end diastolic diameter
LVEF	Left ventricular ejection fraction

LVESD	Left ventricular end systolic diameter
LVFS	Left ventricular fractional shortening
LVSD	Left ventricular systolic dysfunction
LVSP	Left ventricular systolic pressure
mAb	Monoclonal antibody
Mac-3	Macrophage marker M3/84
MaFGF	Mitogenic acidic fibroblast growth factor
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MCL	Micheliolide
MeOH	Methanol
MI	Myocardial infarction
M-Mode	Motion mode
MRI	Magnetic resonance imaging
MS	Mass spectroscopy
MUGA	Multigated acquisition scan
NAC	N-acetylcysteine
NACA	N-acetylcysteine amide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCCTG-N-9831	North Central Cancer Treatment Group Trial N9831
NDF	Neu-differentiating factor

NF-κB	Nuclear factor kappa B
NH_2	Amide functional group
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS3	Nitric oxide synthase 3
NP	Nanoparticles
NPI	Nottingham Prognostic Index
NRG	Neuregulin
NRG-1	Neuregulin protein 1
NS	Non-significant
NSABP B-31	National Surgical Adjuvant Breast and Bowel Project Trial B-31
NYHA	New York Heart Association
N_2	Nitrogen
OH	Hydroxyl functional group
OS	Oxidative stress
OSR	Overall survival rate
OxPC	Oxidized phosphatidylcholine
O_2	Oxygen
PAD	Peripheral artery disease
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen

PER	Pertuzumab
PET	Positron emission tomography scan
PGA_2	Prostaglandin A_2
PGB_2	Prostaglandin B_2
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin F_{2alpha}
PGJ_2	Prostaglandin J_2
PI3K/AKT	Phosphatidylinosital 3-kinase/protein kinase B
p-JNK	Phosphorylated c-Jun N-terminal kinase
PKC	Protein kinase-C
PLAX	Parasternal long axis
p-MAPK	Phosphorylated mitogen-activated protein kinase
PPAR	Peroxisome proliferator-activated receptor
PPAR-α	Peroxisome proliferator-activated receptor alpha
PPAR-δ	Peroxisome proliferator-activated receptor delta
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PPRE	Peroxisome proliferator response element
PSAX	Parasternal short axis
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidene fluoride
PWT	Posterior wall thickness

P38	p38 mitogen-activated protein kinase
Q-TOF	Quadrupole time-of-flight
RAS	Renin-angiotensin system
Ras-Raf-MAPK	Ras-Raf mitogen-activated protein kinase
RC	Regular chow
RDI	Reference daily intake
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPT	Renal proximal tubule
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SBP	Systolic blood pressure
Sch B	Schisandrin B
SD	Standard deviation
SDG	Secoisolariciresinol diglucoside
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SECO	Aglycone metabolite
SEM	Standard error mean

SERM	Selective estrogen-receptor response modulator
sFas	Soluble fas
SH	Thiol/Sulfhydryl functional group
SOD	Superoxide dismutase
SOD2	Superoxide dismutase 2
SR	Strain rate
STAT	Signal transducer and activator of transcription
STAT1	Signal transducer and activator of transcription 1
STAT2	Signal transducer and activator of transcription 2
STAT6	Signal transducer and activator of transcription 6
TBST	Tris buffered saline tween 20
TDI	Tissue Doppler imaging
TGF-α	Transforming growth factor alpha
TNF-α	Tumor necrosis factor alpha
Topo-II	Topoisomerase-II
TP53	Tumor protein 53
TRZ	Trastuzumab
TTE	Transthoracic echocardiography
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling
TVI	Tissue velocity imaging
TXB2	Thromboxane B2
VCAM-1	Vascular cell adhesion molecule-1

V_{ENDO}	Endocardial velocity
WT	Wild type
2D	2-dimensional
5-FU	5-Fluorouricil
6k $PGF_{1\alpha}$	6-keto prostaglandin $F_{1\alpha}$
8,9-DiHETrE	8,9-dihydroxyeicosatrienoic acid
11,12-DiHETrE	11,12-dihydroxyeicosatrienoic acid
12-HHTrE	12-hydroxyheptadecatrienoic acid
16-HETE	16-hydroxyeicosatetraenoic acid
18-HETE	18-hydroxyeicosatetraenoic acid

Chapter 1: Introduction

1.1 Breast Cancer: Epidemiology

According to the Canadian Cancer Society, it is expected that nearly 1 in every 2 individuals will be diagnosed with a form cancer in Canada during their lifetime.[1] Breast cancer is the most commonly diagnosed cancer among Canadian women accounting for 25% off all new cases in 2018.[1, 2] In 2019, it is estimated that 12.5% of women will be diagnosed with breast cancer equating to 72 new diagnoses every day or 3 new diagnoses every hour.[2] In a special report released by the Canadian Cancer Statistics, 47% of new diagnoses of breast cancer in 2018 were classified as stage I with only 5% of cases being stage IV.[2] Currently the five-year net survival rate of female breast cancer is relatively high at 87%; however, survival varies considerably by stage.[2] For instance, while stage I breast cancers have close to a 100% chance of survival, the survival rate of stage IV breast cancers is only 22%.[2] However, the fact remains that with over 5,000 deaths per year, women with breast cancer have a 1 in 5 chance of dying from the disease, making it the second highest cause of cancer related death in Canada.[1, 3] As many as 1 in 8 Canadian women will be affected by breast cancer in her lifetime.[1]

1.2 Breast Cancer: Early Detection and Diagnosis

Since the late 1980's, there have been many major advancements in the detection and prevention for high risk breast cancer patients.[1] While routine physical examination is a part of the initial screening process, the use of full-field digital mammography, magnetic resonance imaging (MRI), ultrasonography, and positron-emission tomography (PET) scan have been added to this regime to enable the detection of smaller tumors at earlier stages of the disease.[4-6] Among these imaging parameters, digital mammography is the most widely used modality for the early detection of

breast cancer. Specifically, the American Cancer Society recommends that: i) women between the ages of 40 – 44 should have the option of starting annual breast cancer screenings with a mammogram; ii) women between the ages of 45 – 54 should be screened with a mammogram once every year; and iii) women 55 years and older should switch to a mammogram screening once every two years.[7] However, if there is a family history of breast cancer, which is defined as: i) a first degree relative diagnosed with breast cancer prior to the age of 50 years old; ii) a first degree relative with cancer in both breasts that was diagnosed at any age; or iii) two or more first degree relatives with breast cancer who were diagnosed at any age, a women should undergo yearly mammograms beginning no later than ten years before the age of the earliest diagnosis in the family (not earlier than age 25 or later than age 40) for early detection of breast cancer.[8] In addition to a family history of breast cancer, women who are at an above average risk for breast cancer include a history of atypical hyperplasia, lobular carcinoma in situ, mantle radiation, or who possess a genetic predisposition (i.e. Breast Cancer gene (BRCA) mutation) may undergo supplemental imaging including an annual breast MRI, beginning at the age of 25 years old.[8]

The institution of these screening protocols has decreased the incidence of morbidity and mortality of breast cancer over the past three decades. Specifically, these novel imaging techniques have benefited Canadian women by preventing over 32,000 breast cancer related deaths between 1987 and 2012.[1 4, 5, 9, 10] In addition to these imaging techniques, recent advancements in the management of breast cancer patients now includes screening individuals for predisposing genetic factors. Studies conducted by Pasche *et al.* (2008) and Lalloo *et al*. (2003) elucidated that there is a strong correlation between a family history of breast cancer and mutations in the breast cancer 1 (BRCA1), breast cancer 2 (BRCA2), and tumor protein 53 (TP53) genes.[11, 12] The Lalloo *et al*.

(2003) study concluded that women are at a three to four times increased risk of developing breast cancer in their lifetime if they carry a mutation in one of the BRCA genes.[12] In order to determine if a women carries a mutation in any of these genes, a blood sample is collected from the patient and a deoxyribonucleic acid (DNA) analysis is performed to identify any harmful mutations.[13]

Despite the numerous imaging modalities available to detect breast cancer, a breast core needle biopsy is required to make a definitive diagnosis as it provides information on tumor aggressiveness and proliferative activity.[14] A tissue biopsy is performed by inserting a needle into the breast lump and testing the tissue sample for the presence of cancerous cells.[15] In 1982, Haybittle *et al.* created the Nottingham Prognostic Index (NPI) (Equation 1) for histological grading of primary breast cancers.[16] This was a retrospective, multivariate study which investigated several morphological features including: age, menopausal status, tumor size, lymph node involvement, tumor grade, cellular reaction, presence of sinus histiocytosis in lymph nodes, and estrogen receptor (ER) content in 387 patients with primary, operable breast cancer.[16] This study identified that three significant factors including the size of the index lesion (in centimeters), the node status (0 nodes = 1, 1 – 3 nodes = 2, and >3 nodes = 3), as well as tumor grade (grade I = 1, grade II= 2, and grade III = 3), were significantly related to patient survival.[16] The NPI was then validated by Elston and Ellis in 1991, who confirmed that the higher the index the worse the prognosis.[17] The Nottingham Prognostic Index remains a standard prognostic index for all patients with breast cancer and is routinely used in practice.

Equation 1: The Nottingham Prognostic Index.

$$\text{Nottingham Prognostic Index (NPI)} = [0.2 \times \text{Size (cm)}] + \text{Node Status} + \text{Tumor Grade}$$

1.3 Breast Cancer: The Treatment Plan

At the beginning of the 21st century, the initial approach to therapy for breast cancer was based on the premise that the disease metastasized in an orderly fashion which would then proliferate throughout the body.[18] Thus, the accepted method for treatment was aggressive surgery via a radical mastectomy.[19] Subsequent randomized trials indicated that there was no benefit between a radical mastectomy compared to a less aggressive surgery such as lumpectomy.[20, 21] However, reoccurrence of the disease remained a serious clinical issue irrespective of the primary surgical intervention.[20, 21] Surgical procedures began to shift from a more aggressive (radical mastectomy) to a less aggressive lumpectomy or lymphadenectomy where a small tumor and ring of tissue or one group of lymph nodes are removed, as opposed to the complete removal of the affected breast.[6]

Radiation therapies are most commonly used after a surgical procedure. This therapy uses targeted high energy X-rays to potentially kill any of the remaining cancerous cells in or around the breast after surgery. Typically, radiation therapy involves 5 sessions per week for a total duration of 3 to 7 weeks. Each of these treatment sessions are approximately 10 to 30 minutes in length. Radiation therapy is spread out in these short sessions over the course of several weeks to allow for the recovery of the healthy cells around the target area. Radiation therapy has been proven to be beneficial for overall patient outcome as well as reducing the risk of local recurrence by 16% of patients.[22, 23]

Local therapy continued to evolve from a more aggressive to less aggressive regimen as the types of adjuvant systemic therapies and their indications expanded.[18] The driving force behind this shift in practice was the recognition and identification of different prognostic and predictive factors.[18]

Specifically, women with breast cancer were evaluated on several criteria including: i) the Nottingham Prognostic Index of tumor size, lymph node involvement, and tumor grade; as well as ii) the expression of estrogen, progesterone and human epidermal growth factor receptors (HER2). [16, 24, 25] For example, overexpression of ER-α located on chromosome 6q25.1 increases the risk of breast cancer development by a factor of 1.5 in women.[26]

The evaluation of these two criteria were the driving force behind the development of adjuvant systemic chemotherapy, endocrine therapy, and anti-HER2 directed therapy, which lead to a series of clinical trials evaluating their efficacy.[27-29] These studies concluded that adjuvant systemic chemotherapy, endocrine therapy, and anti-HER2 directed therapy substantially reduced the risk of recurrence and improved overall survival when added to surgical intervention.[27-29] The 1952 National Surgical Adjuvant Breast and Bowel Project (NSABP) B-01 Trial reported that the adjuvant alkylating agent thiotepa significantly decreased recurrence in pre-menopausal women when administered after radical mastectomy.[27] A study from 1975 indicated the same benefit with the alkylating agent L-phenylaline mustard.[28] Another trial from the Istituto Nazionale Tumori in Milan, Italy reported that the combination of Cyclophosphamide, Methotrexate, and 5-Fluorouricil (CMF) significantly reduce the risk of recurrence.[29] This trial was at the forefront of modern age adjuvant polychemotherapy regimens that are commonly used in today's oncological practice.

There are two common first generation polychemotherapeutic regimens that are typically used to treat breast cancer. The first regimen of chemotherapy is a mixture of Adriamycin and Cyclophosphamide (AC).[1] AC chemotherapy is administered on a bi-weekly basis for 4 – 6 treatment cycles with routine dosages of 60 mg/m^2 Adriamycin (A) and 600 mg/m^2

Cyclophosphamide (C).[1] Interestingly, a study by Shulman *et al.* (2014) found no advantage in a longer compared to a shorter administration period of AC treatment in women with breast cancer.[30] The NSABP-11 trial was the first to evaluate a Doxorubicin (DOX) based chemotherapy in a population of non-tamoxifen responsive patients, which discovered an improved 5-year disease free survival (DFS) and a trend towards improved overall survival rate (OSR).[31] The NSABP-15 trial went on to test the same chemotherapy regimen on node-positive breast cancer discovering similar 3-year DFS and OSRs to the original CMF chemotherapy.[32] Finally, the NSABP-25 trial showed no difference in outcome in node-negative patients treated with CMF or AC.[33]

The second and most commonly used chemotherapy regimen that is used to treat breast cancer is composed of 5-Flurouroucil (5-FU), Epirubicin (E; DOX), and Cyclophosphamide (C).[1] FEC chemotherapy is administered every 21 days for a total of six treatment cycles.[1] The cumulative dose of FEC chemotherapy is 500 mg/m^2 5-FU, 100 mg/m^2 E, and 500 mg/m^2 C.[1] FEC chemotherapy is advantageous as it is classified as a chemoendocrine therapy.[34] The French Adjuvant Study Group (FASG) compared a tamoxifen only treatment with an Epirubicin-based chemoendocrine therapy in 457 post-menopausal women with ER+ breast cancer.[34] This study found that the 9-year DFS and OSRs trended in favour of the latter treatment group.[34] There are several other second (FEC100; CAF/FAC: C, DOX, 5-FU; AC-T; DOX, C, and Paclitaxel, etc.) and third (DAC: Docetaxel, DOX, C; Sequential FEC-T: FEC and Docetaxal; and AC-T: dose dense sequential AC-T, etc.) generation chemotherapy regimens that are available to patients. However, the choice of chemotherapy regimen is individualized based upon disease-specific factors including prognostic factors, predictive factors, the underlying risk of recurrence, as well as the projected relative and absolute benefits from chemotherapy.[18]

In addition to standard chemotherapy, endocrine therapies were developed to specifically treat estrogen receptor and progesterone receptor positive breast cancers. [35] There are multiple hormone treatment options for ER+ breast cancer. These include: i) Luteinizing hormone-releasing hormone agents (LHRHs); ii) Aromatase inhibitors; iii) Selective estrogen-receptor response modulators (SERMs); and iv) Estrogen-receptor downregulators (ERDs).[36] LHRHs (Zoladex, Lupron, and Trelstar) function by inhibiting the ovarian production of estrogen, which decreases the total amount of estrogen the tumor can access for growth.[36] This treatment is typically used for premenopausal women with early stage ER+ breast cancer.[36] In comparison, Aromatase inhibitors including Arimidex, Aromasin, and Femara are prescribed to postmenopausal women to reduce the availability of estrogen.[36] Aromatase inhibitors exert their effect by blocking the enzyme aromatase, which is responsible for the conversion of androgen into estrogen.[36] Tamoxifen is the most widely used SERM to treat ER+ in a variety of patients. Tamoxifen binds to the estrogen receptors that are present on the breast cells.[36] This binding prevents an estrogen signal cascade that would otherwise upregulate tumor cell proliferation. Similarly, ERDs like Faslodex interfere with the estrogen binding receptors. Additionally, Faslodex works to reduce the total number of estrogen receptors present on the breast cells.[36]

Finally, recent advancements in our understanding of Human Epidermal Growth Factor Receptor 2 positive (HER2+) breast cancers has led to the development and use of monoclonal antibodies. Originally, Trastuzumab (TRZ; Herceptin) was jointly developed by Genentech Inc. (South San Francisco, CA) and the University of California, Los Angeles (UCLA) in the early 1990's. In 1998, Pegram *et al.* performed a Phase II clinical trial, which investigated the use of TRZ as an anti-cancer agent in patients with HER2+ breast cancer.[37, 38] These patients were injected with a loading

dose of TRZ (250 mg i.v.) at baseline and received weekly doses of 100 mg i.v. for 9 weeks thereafter.[38] This study concluded that TRZ: i) resulted in higher response rates in HER2 overexpressing metastatic breast cancers; ii) had no apparent increase in toxicity; and iii) TRZ's pharmacology was unaffected by the co-administration of Cisplatin.[38] Due to the success of this and several other clinical trials involving the use of TRZ to treat HER2 positive breast cancers, the drug was fast-tracked by the Food and Drug Association (FDA) and gained approval in September of 1998.[37, 39-42] Since its approval, TRZ continues to be widely used in cancer centres across the globe in the management of women with HER2 positive breast cancer.

Recently, a second monoclonal antibody (mAb) Pertuzumab (PER) was developed to treat HER2+ breast cancer. PER was created at the same time as TRZ by Genentech Inc, in the early 1990's. In 2012, the first positive results were published by the Clinical Evaluation of PER and TRZ (CLEOPATRA) study group.[43, 44] This group performed a randomized placebo-controlled Phase III clinical trial where PER was administered to HER2+ metastatic breast cancer patients in combination with TRZ and Docetaxel.[43, 44] Shortly after the CLEOPATRA Trial was published, PER gained FDA approval in 2012 for the treatment of HER2+ metastatic breast cancer.[45] Additionally, the results of the Phase II NeoSphere Trial, which investigated the use of PER in the neoadjuvant setting was published later that year, receiving FDA approval in 2013.[46,47] In today's practice, the diagnosis and treatment for breast cancer is multifactorial, involving a combination of: i) imaging modalities; ii) genetic screening protocols; iii) surgical interventions; iv) radiation therapies; and v) pharmacological agents. Fortunately, the combination of these tools has contributed to an overall decrease in morbidity and mortality in this patient population.[48]

1.4 Breast Cancer Therapy and Cardiotoxicity

In Canada, both cancer and cardiovascular disease are the leading causes of mortality, accounting for over 120,000 deaths on an annual basis.[1, 49] Cardio-Oncology is an evolving discipline that focuses on the prevention, diagnosis, and management of cancer patients who are at an increased risk of developing cardiovascular complications due to their anti-cancer treatment. Although the current combination of surgical resection, radiation therapy, and chemotherapy are effective in prolonging the survival of women with breast cancer, the administration of anti-cancer agents, in particular DOX, is associated with an increased risk of developing chemotherapy induced cardiotoxicity. The addition of TRZ to breast cancer therapy further compounds this issue of drug induced heart failure. Although DOX+TRZ decreases the risk of recurrence and death in the breast cancer setting, a major side effect of this therapy is the 1 in 4 risk of developing heart failure, affecting over 8000 Canadian women annually.[1, 2] Therefore, the need for an effective protective regime to attenuate drug-induced cardiotoxicity associated with breast cancer therapy is both acute and overarching.[50-52]

Thus, basic scientists, cardiologists, and oncologists have placed an emphasis on determining the underlying mechanisms of chemotherapy induced cardiotoxicity. Several collaborative basic science and clinical studies have been performed by our cardiovascular imaging laboratory over the past decade to evaluate: i) the prevalence of chemotherapy mediated cardiotoxicity; ii) the role of cardiac imaging in its early diagnosis; and iii) the role of anti-oxidants and heart failure medications in the prevention of this disease.[53-66] Recently, we have placed a larger focus on the inflammatory and oxidative stress (OS) mechanisms related to chemotherapy mediated cardiotoxicity. [54-57, 60, 62, 65, 67-77] Many of our studies have revealed that the production of

chemotherapy-induced free radicals upregulates the OS pathway, which plays a vital role in damaging cardiomyocytes. However, only a few studies have investigated the influence of the inflammatory pathway on this drug induced cardiotoxicity.[70-72]

Given the proposed mechanisms of damage, recent efforts to attenuate chemotherapy induced cardiac dysfunction has led to investigations using prophylactic anti-oxidants and heart failure medications.[78-80] However, the addition of anti-oxidants and heart failure medications to this treatment regime pose their own health and economic concerns. For example, adverse side effects of vasodilatory drugs including angiotensin converting enzyme (ACE) inhibitors, beta-adrenergic receptor blockers (β-blockers), and renin-angiotensin system (RAS) antagonists, which have all been approved and are used for treatment of chemotherapy induced cardiotoxicity include: arrhythmia, hypotension, hyperkalemia, dizziness, headache, weakness, and nausea.[78-81] Additionally, the cost and/or administration of these medications can be a burden to patients. Fortunately, a natural, cost-effective solution to this problem may potentially exist.

In women with breast cancer, complementary and alternative medicine approaches are widely used in an attempt to reduce the overall burden of the disease and prevent recurrence. Functional foods are defined as having a potentially positive effect on overall health beyond their basic nutritional components. Interestingly, functional foods have been reported to provide significant health benefits in several disease conditions including cancer and cardiovascular disease.[82] Approximately 30% of women with breast cancer consume alternative medicine supplements, including flaxseed (FLX), to treat their underlying cancer and to improve their overall health.[83] FLX is a non-toxic whole grain composed of high concentrations of alpha-linolenic acid (ALA)

and is an essential source of plant lignans including secoisolariciresinol diglucoside (SDG), which have potent anti-inflammatory, anti-oxidant, and anti-fibrotic properties.[84-87] Coincidentally, Manitoba is one of the top FLX producing regions in the world, which has contributed to the increase in local public awareness of the importance of the consumption of this whole grain commodity for disease prevention. However, the specific cardioprotective role of FLX in the prevention of DOX+TRZ induced cardiotoxicity has yet to be investigated.

Chapter 2: Literature Review

2.1 Anthracyclines: A History Lesson

In the 1930's, anthracyclines were used as antibiotics.[88] However, during the 1960's, the chemical properties of anthracyclines were re-characterized and it was discovered that they also had potent anti-cancer properties.[88] This discovery led to the classification of anthracyclines as a chemotherapeutic agent, which would later progress into being one of the most commonly used forms of anti-cancer medications available. Daunorubicin (DNR) and DOX are the two anthracycline-based drugs that were created from isolations taken from the pigment producing soil bacterium *Streptomyces peucetius var caesius.*[88, 89] Since then, it was revealed that DNR and DOX possess a broad range of anti-cancer properties, which are used to treat human carcinomas.[89, 90]

The chemical structures of DNR and DOX are identical only differing by a single functional group on Carbon-14 at the terminal chain. At this terminal position, a methyl group ($-CH_3$) is linked to Carbon-14 in DNR (Figure 1) where as a hydroxyl group (-OH) is bound to this carbon in DOX forming a primary alcohol (Figure 2).[51, 91] This small, yet significant structural change between these two drugs results in dramatic differences in chemical properties. The addition of a hydroxyl group to DOX increases its' lipophilicity, enabling DOX to cross the cellular lipid bilayer and enter the cytosol.[92] As a result of DOX's trans-membrane capabilities, the half-life of DOX increases within the body.[92] DOX is the key chemotherapeutic component used in the treatment of breast cancer. However, the use of DOX as well as that of DNR has been extended to treat other cancers including childhood solid tumors, soft tissue sarcomas, and lymphomas.[88, 93, 94]

Figure 1: The chemical structure of Daunorubicin (7S,9S)-9-acetyl-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione.

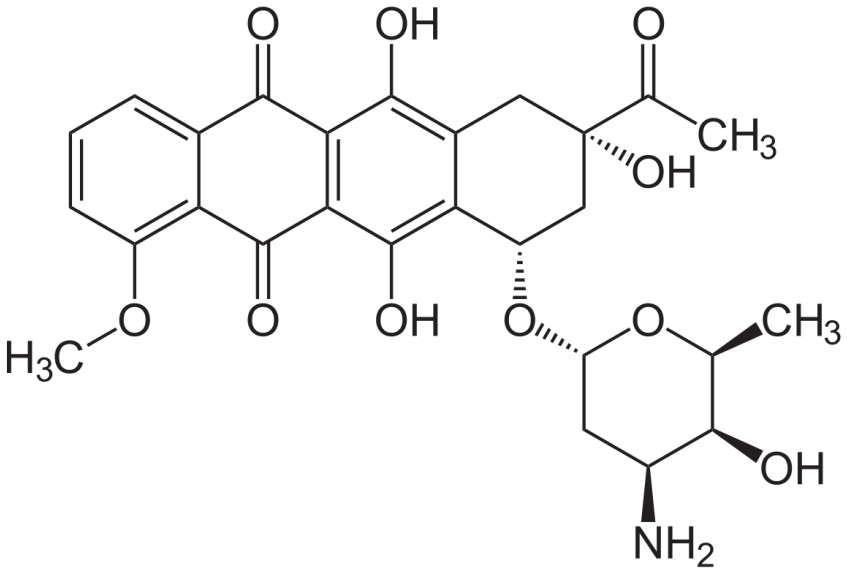
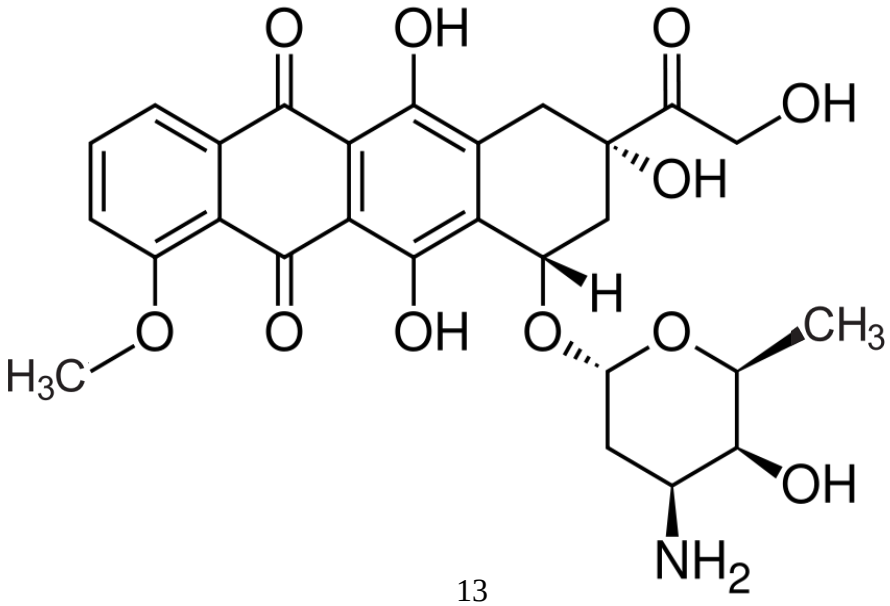


Figure 2: The chemical structure of Doxorubicin (7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione.



2.2 The Anti-Cancer Mechanisms of Doxorubicin

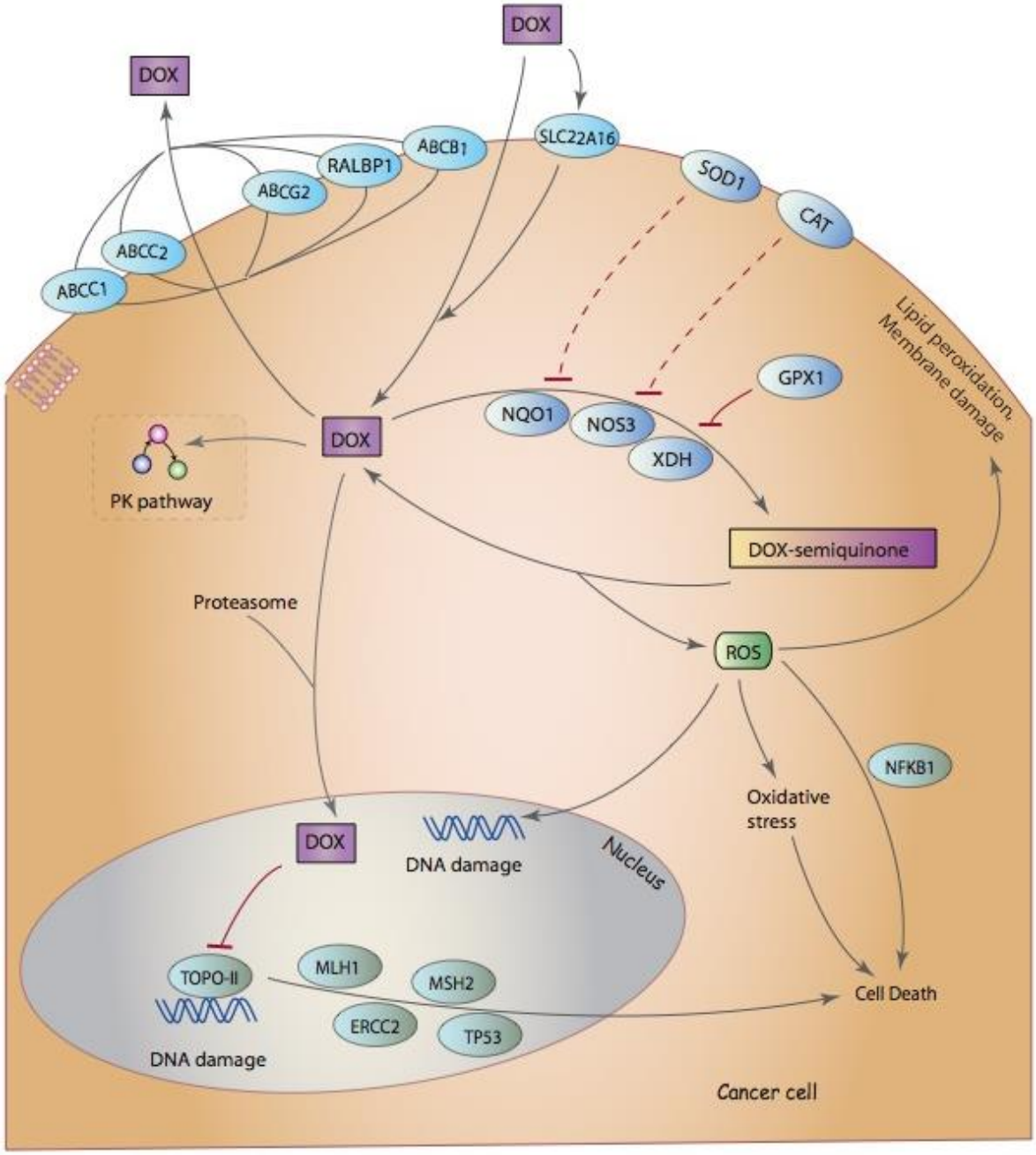
There are multiple mechanisms in which DOX exhibits its anti-tumor activity, which results in a reduction in the size of solid tumors. DOX's anti-cancer capabilities are a direct result of its ability to intercalate between DNA base pairs, which effectively inhibits replication and proliferation of the otherwise rapidly dividing tumor cells.[51] Modes of action of DOX include: DNA crosslinking, direct membrane damage, altered DNA binding, alkylation, histone eviction, free radical generation, as well as inhibitory actions on the enzyme topoisomerase-II (Topo-II) (Figure 3).[51, 92, 93, 95-98] Of these mechanisms, free radical generation and Topo-II inhibition are the two most commonly accepted and best characterized modes of anthracycline function.[51, 92, 96]

Anti-tumor effects of DOX induced by reactive oxygen species (ROS) are generated through non-protein dependent DNA strand breaks.[93] The generation of ROS involve a sequence of redox cycling reactions, which begins with DOX's quinone structure acting as an electron acceptor.[95] This process produces a semi-quinone free radical.[94, 95] In anaerobic conditions, this free radical is stable. However, the unpaired electrons are donated to oxygen (O_2) in an aerobic environment, which generates an unstable superoxide radical instead.[95] Once in contact with DNA, these superoxide radicals induce single or double stranded DNA breaks, which inhibit cellular replication and proliferation.[95] Additionally, DOX can bind iron to form a complex, which produces partially reduced forms of O_2 that behave in a similar fashion.[94] Superoxide dismutase (SOD), dimethyl sulfoxide (DMSO), catalase, and anti-oxidants are capable of preventing the activation of Poly (ADP-ribose) polymerase (PARP) through the inhibition of DNA strand damage. Furthermore, DOX proficiently reduces the levels of glutathione peroxidase (GHS), a

known free radical scavenging protein.[69] Therefore, we can conclude that free radical formation indeed plays a role in the anti-tumor efficiency of DOX.

Unlike the free radical formation mechanism of DOX proposed above, topoisomerase-II (Topo-II) promotes protein-dependent cleavage of DNA.[99] Topo-II is an enzyme that functions by introducing or removing DNA supercoils thereby changing the topological state of DNA.[99] This directly impacts the processes of DNA replication and transcription.[99] Topo-II's direct effect on DNA and cellular proliferation has made it a key target of anti-cancer therapies. DOX arrests uncontrolled tumor growth by inhibiting mammalian cell DNA as well as ribonucleic acid (RNA) synthesis.[51] As double stranded DNA unwinds from the alpha-helix configuration, an increased amount of strain is exhibited due to DNA supercoiling.[99] Under normal physiological conditions, Topo-II will cleave the double stranded DNA to relieve this strain by allowing the supercoiled segments to pass through the breaks.[99] Topo-II will then ligate the DNA strands back together to re-form the original DNA molecule.[99] This process allows the DNA to relax and continue to unwind for the replication process.[99, 100] DOX forms a cleavable complex which stabilizes Topo-II through DNA intercalation.[101] This complex inhibits the enzymatic function of Topo-II by preventing the DNA from being re-annealed leaving lesions within the unwinding strand of DNA. Eventually, this results in programmed cell death, which inhibits further tumor cell replication and proliferation.[101, 102] The multiple anti-tumor properties of DOX including the mechanisms involved in free radical formation and Topo-II inhibition establish DOX's efficacy as an anti-cancer agent.

Figure 3: Doxorubicin pathways: pharmacodynamics and adverse effects. Adapted from Thron et al. (2011).[98]



2.3 The Clinical Use of Doxorubicin

The clinical use of DOX is advantageous as it can be used to treat both early and advanced stage breast cancers. The versatility of DOX is attributed to its multiple anti-cancer mechanisms.[51, 92, 93, 95-97] DOX has the capacity to exert its affect in three specific courses. In the first case scenario, a patient may be administered DOX after surgery (i.e.: radical mastectomy, lumpectomy, or axillary lymphadenectomy) to reduce the risk of recurrence.[1] Comparatively, DOX may be administered prior to surgery to shrink large advanced stage breast cancer tumors.[1] Finally, DOX may also be given to treat advanced-stage breast cancers.[1] In order to exert its effect, DOX is administered via an intravenous injection (i.v.) through a central or peripheral line in the venous line. A dose of 40 – 60mg/m^2 is administered over several minutes once every three weeks for a total of 6 to 8 treatment cycles.[1] Additionally, DOX may be administered by continuous infusion through a central catheter. However, it is important to note that a lifetime cumulative dose of DOX cannot exceed 500 mg/m^2 of body surface area as it will place a patient at an unacceptable risk of developing cardiotoxicity.[91, 92, 103]

Typically, DOX is administered in combination with other chemotherapeutic agents. Specifically, DOX is administered as part of AC or FEC chemotherapy regimens. Adriamycin and Cyclophosphamide (AC) chemotherapy is administered once every two weeks for four or six treatment cycles with routine dosages of 60 mg/m^2 Adriamycin (A; DOX) and 600 mg/m^2 Cyclophosphamide (C).[1] Comparatively, FEC is the most commonly used combination chemotherapy regimen in the treatment of breast cancer.[1, 2] FEC is composed of 5-Flurouroucil (5-FU), Epirubicin (E; DOX), and Cyclophosphamide (C), and is administered every 21 days for a total of six treatment cycles.[1] The cumulative dosages of FEC chemotherapy are 500 mg/m^2 of 5-

Fluorouricil (5-FU), of 100 mg/m^2 Epirubicin (E; DOX), and of 500 mg/m^2 Cyclophosphamide (C).[1] In addition to breast cancer, DOX may be used to treat other cancers including: bladder, stomach, lung, ovarian, thyroid, soft tissue sarcoma, multiple myeloma, leukemias, and Hodgkin's lymphoma.[1]

2.4 The Human Epidermal Growth Factor Receptors

The Human Epidermal Growth Factor Receptors (ErbB/HER/c-*neu*) are a subset of receptor tyrosine kinases (RTK) from the epidermal growth factor receptor family.[104, 105] RTK's are responsible for mediating cell growth, differentiation, as well as cell survival.[104, 105] Signaling between these receptors is interdependent and involves a cellular network.[105, 106] The ErbB subset of RTK consists of four closely related isoforms: HER1, HER2, HER3, and HER4. These isoforms are expressed within the myocardium of the fetal and adult heart of both rodents and humans.[107, 108] These RTK isoforms are cell surface receptors that are comprised of three distinct domains: i) a single membrane-spanning domain; ii) a ligand activated RTK domain; and iii) a carboxy-terminal regulatory domain.[109, 110] Additionally, these receptors are capable of acting as a co-receptor in neuregulin (NRG) signaling by coupling to a unique intracellular signaling pathway.[108, 111]

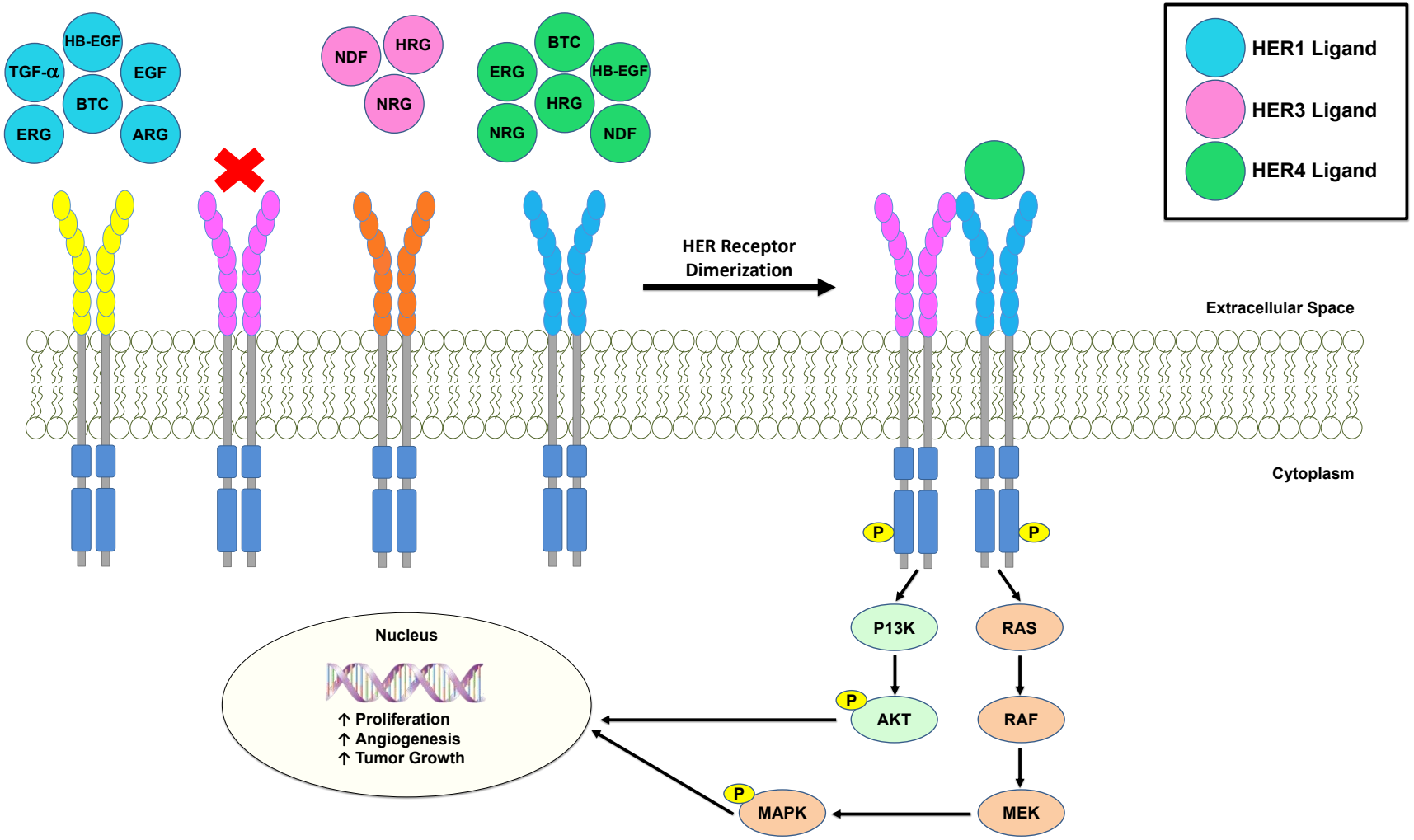
The ErbB receptor pathways are initiated by a specific ligand-receptor interaction, which takes place on the extracellular domain of the receptor. Most ligands binding to this receptor family are highly specific to one of the receptor isoforms, but there are some ligands that bind to pairs of receptors due to their homology.[112] For example, epidermal growth factor (EGF), transforming

growth factor alpha (TGF-α), and amphiregulin (ARG) are specific to HER1; *neu*-differentiating factor (NDF), neuregulins (NRG), and heregulins (HRG) bind to both HER3 and HER4; betacellum (BTC), epiregulin (ERG), and heparin-binding EGF-like growth factor (HB-EGF) bind to HER1 and HER4 isoforms.[110, 112] In comparison, there are no ligands that bind directly to the HER2 isoform. Instead, HER2 acts as a common receptor for all other isoforms through heterodimerization.[108, 111]

Within the breast cancer setting, ErbB2, also known as HER2 or c-*neu* is of specific importance. While the extracellular domain of HER2 is ligand independent, it can be transactivated by G-protein coupled receptors (GPCR) and regulated through positive intracellular signaling of matrix metalloproteinases and Src.[58, 113, 114] Ligands such as cytokine interleukin-6 (IL-6) and NRG stimulate HER2 to dimerize with adjacent HER receptor isoforms. Once in a dimerized structure, cellular proliferation, angiogenic, and survival pathways are initiated through the Ras-Raf-mitogen-activated protein kinase (Ras-Raf-MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathways, respectively (Figure 4).[109] Overexpression of the HER2 receptor increases it's intensity and influence on these aforementioned pathways.[115] Protein kinase-C (PKC) activating growth factors and hormones lead to a reduction in the levels of phosphorylated tyrosine, while HER2 activation and transmodulation are driven by intermediate signaling sequences of the epidermal growth factor receptor (EGF-R).[116] The overexpression of the HER2 receptor directly affects the degree of crosstalk between adjacent receptors and other signaling pathways ultimately leading to uncontrolled cellular proliferation.[106, 114] Additionally, direct implications in cellular OS and apoptosis have been showcased between HER2 and EGF-Rs

through mitogenic signaling pathways, both of which are key components in anti-HER2-induced cardiomyopathy.[117]

Figure 4: Heterodimerization of the human epidermal growth factor receptor 2 (HER2) with adjacent receptors (HER1, HER3, HER4) and the downstream activation of the P13K and Ras-Raf-MAPK pathways.



2.5 Breast Cancer and Over Expression of HER2

It has been well established that a number of carcinomas overexpress the human epidermal growth factor receptor (ErbB/HER/c-*neu*) family of transmembrane tyrosine kinase receptors (RTK).[118-120] Approximately 1 in every 4 breast cancers overexpress the HER2 receptor.[1, 121, 122] Specific forms of breast cancer such as ductal carcinoma in situ (DCIS) significantly overexpress HER2 in 90% of cases.[123] Overexpression of HER2 also known as HER2 positive cancers, typically confer poor prognosis in patients as aggressive tumor growth is promoted, which impedes the efficacy of standard chemotherapies.[121, 124] Breast tissue that is positive for HER2 due to the amplification of the HER2 gene, which is responsible for encoding the HER2 receptor. Overexpression of HER2 leads to an increase in the total number of HER2 receptors present on the cell surface, which accelerates cellular proliferation, angiogenesis, and survival, ensuring an aggressive malignancy.[125, 126] These cells display hyperactivity as they upregulate active cyclin-dependent protein kinase complexes making them resistant to standard chemotherapies and radiotherapy-induced apoptosis.[123, 127] Due to the aggressive nature of these tumors as well as the resistance to cytotoxic agents such as DOX, a lower survival outcome of patients with HER2 positive status was previously clinically accepted.[128]

Early detection of HER2 overexpression is crucial to the patient's outcome as it occurs early on in the progression of breast cancer and requires aggressive treatment, if found to be positive.[124] Overexpression of HER2 is detected through immunohistochemistry (IHC), chromogenic *in situ* hybridization (CISH), and/or fluorescence *in situ* hybridization (FISH).[129-134] The Canadian Cancer Society guidelines most commonly employ the use of IHC and FISH for HER2 status testing.[1] Patients are categorized into HER2 positive or negative status by assessing the recognition

and interaction of monoclonal antibodies with HER2 from tissue biopsy's taken from the patient.[132, 135] The early detection of HER2 positive breast cancers raises several clinically beneficial outcomes, which include a more accurate prediction of prognosis and response to drug regimens including: endocrine therapy, tamoxifen, anthracycline based chemotherapy, and the use of anti-HER2 drugs (ex: TRZ).[136-138]

2.6 Monoclonal Antibodies: The History of Trastuzumab

In 1984, Schechter *et al.* published a novel article in *Nature*, citing the discovering of the *neu* oncogene.[139] Originally, this study was investigating a family of transforming genes from the genomes of rat neuro/glioblastoma cell lines that were derived from ethylnitrosourea (ENU) induced tumors.[139] However, Southern blot analysis of the transformed cells of the rat genomes did not indicate the presence of *ras* oncogenes; rather, there was an independently activated oncogene.[139] This new oncogene was termed *"neu"*. The study went on to validate that: i) *neu* induces the synthesis of the tumor antigen p185; ii) the *neu* oncogene is homologous to the ErbB gene and p185 antigen; and iii) *neu* is serologically related to the epidermal growth factor receptor (EGF-R).[139]

Shortly after these findings were published, Drebin *et al.* (1986) described a monoclonal antibody that was reactive with the surface domain of the p185 antigen.[140] This study found that *in vivo* treatment with an anti-p185 monoclonal antibody significantly inhibited the tumorigenic growth of *neu*-transfromed cells that had been implanted in immunocompromised nude mice.[140] These results demonstrated that a monoclonal antibody that is specific to an extracellular domain of an oncogenic-encoded protein can exert significant anti-tumor properties.[140] Additionally, Drebin *et*

al. (1986) cited that monoclonal antibodies may be useful as future anti-cancer therapies.[140] Based on these data sets, a joint collaboration between Dr. Dennis Slamon, Genentech Inc. (South San Francisco, CA) and the University of California, Los Angeles revolutionized the treatment of HER2+ breast cancers by establishing a HER2 targeted therapy: TRZ.[38] TRZ (Herceptin) is a humanized (mouse) monoclonal antibody whose fragment antigen-binding (Fab) region specifically binds with high affinity to the extracellular domain of the human epidermal growth factor receptor 2 (HER2) as shown in **Figure 5.**[110] It is composed of anti-HER2 light (1 and 2) and heavy (1 and 2) chains and has a molecular mass of 145531.5 g/mol. The specific amino acid light and heavy chain sequences are illustrated below in **Figures 6 and 7.**[141]

Figure 5: Ribbon diagram of the Fab fragment (cyan) of Trastuzumab bound to the extracellular domain of HER2 (gold).[110]

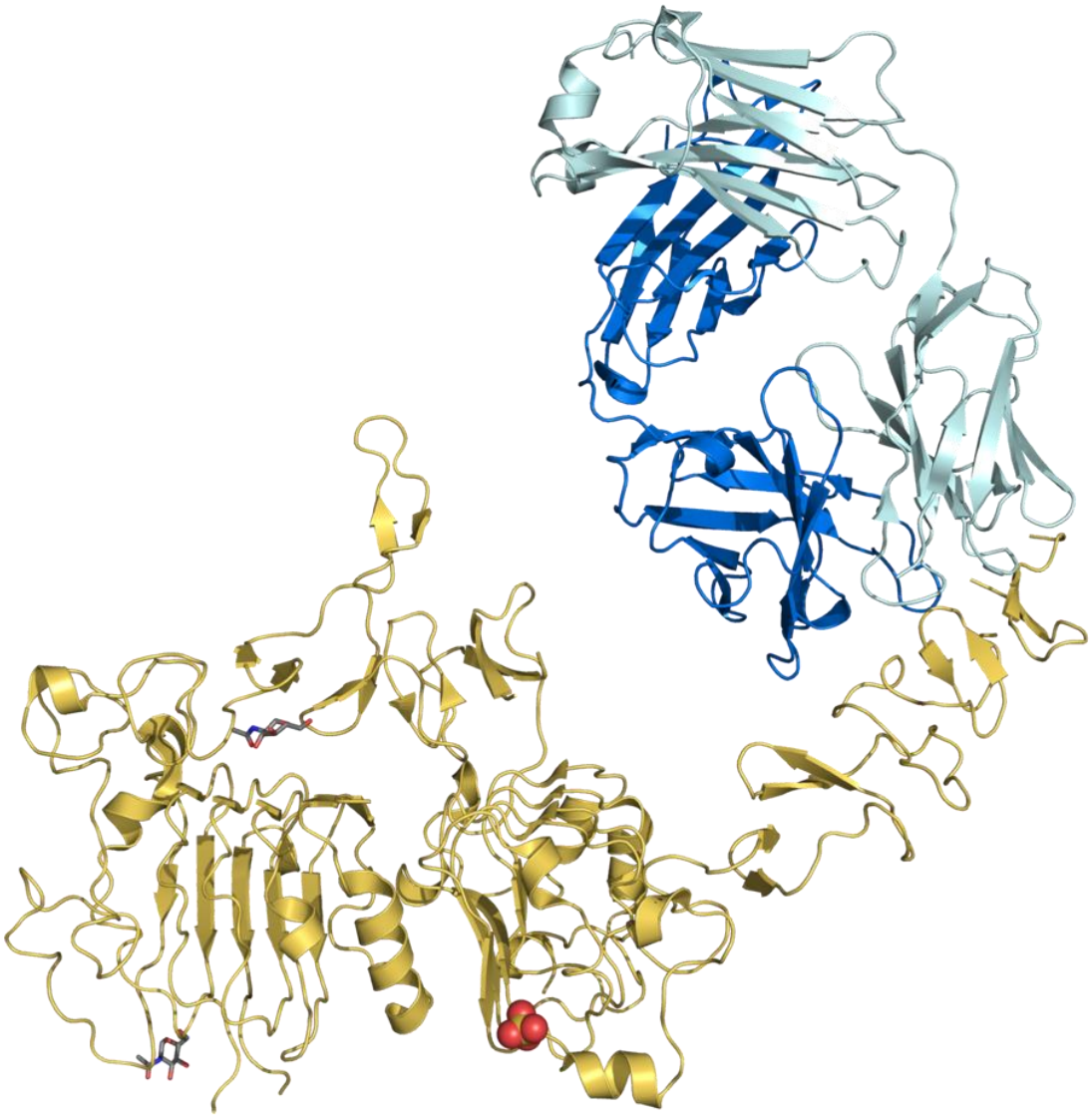


Figure 6: Anti-HER2 Light Chain (1 and 2).[141]

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVP
SRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPS
DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTL
TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

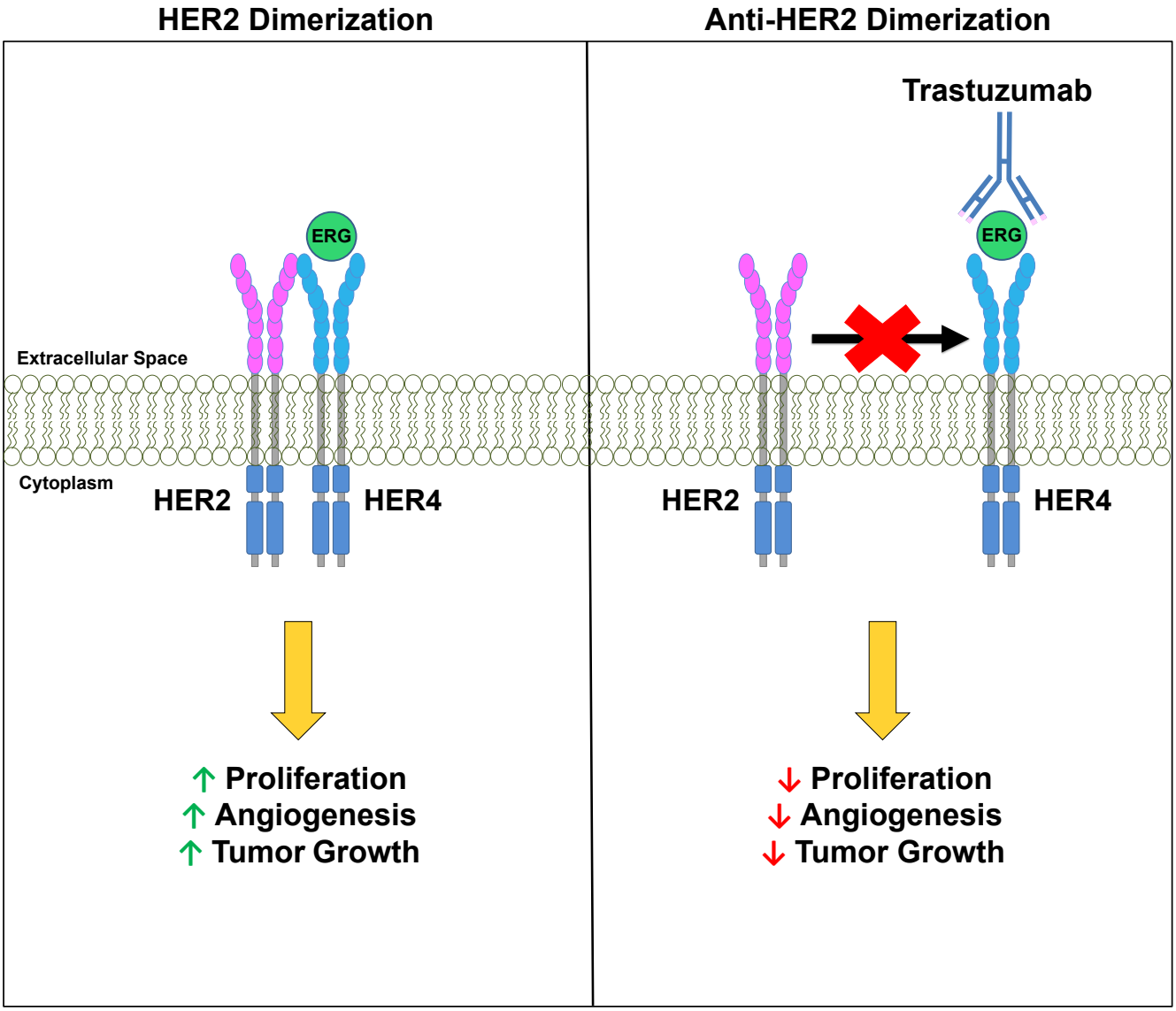
Figure 7: Anti-HER2 Heavy Chain (1 and 2).[141]

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT
RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP
AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA
PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV
YTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS
KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

2.7 Trastuzumab and the Inhibition of HER2

TRZ (Herceptin) is the recommended treatment for HER2 positive breast cancers.[122] TRZ is a monoclonal antibody that is targeted against the HER2 receptors. It functions by directly recognizing and binding to the HER2 receptor, which effectively inhibits the dimerization of HER2 with adjacent HER receptors (Figure 8).[122] Specifically, TRZ binds to the extracellular domain, which interrupts the dimerization process and disrupts the downstream signaling cascade. This interruption in downstream signaling inhibits the Ras-Raf-MAPK and PI3K/AKT pathways, reducing cell survival and proliferation.[106, 122] In addition to the anti-dimerization properties of TRZ, this monoclonal antibody is able to downregulate the expression of HER2 by enhancing its internalization and degradation.[128, 142] Several antibodies specific to HER2 including TRZ, mechanistically reduce the number of cells entering the S-phase of the cell cycle by upregulating cyclin-dependent kinase inhibitors (CDKI) such as $P27^{Kip1}$, as well as the levels of Rb-related protein p130.[105] The binding of TRZ to HER2 overexpressing tumor cells recruits and activates the human immune system through the immunoglobulin G1 Fc domain.[105, 136] As a result of the activation of this domain, TRZ is able to inhibit the HER2 signals that promote cellular survival and uncontrolled cell growth, thus potentiating its anti-cancer effects.[105, 136]

Figure 8: The anti-dimerization process of the human epidermal growth factor receptor 2 (HER2) with adjacent receptors (HER1, HER3, HER4) due to the administration of Trastuzumab.



Administration of TRZ prevents the dimerization of the HER2 and HER4 receptors as TRZ occupies the binding region. Binding of TRZ to the HER4 results in decreased cell proliferation, angiogenesis, and tumor growth. ERG, Epiregulin; HER2, Human epidermal growth factor receptor 2; HER4, Human epidermal growth factor receptor 4; TRZ, Trastuzumab.

2.8 The Clinical Use of Trastuzumab

TRZ can be used clinically both in the adjuvant and metastatic settings of breast cancer. [39, 121, 122, 143] In the adjuvant setting, TRZ is administered after the completion of anthracycline based chemotherapy. A loading dose of 8 mg/kg is first administered with a weekly maintenance dose of 6 mg/kg every three weeks for a total of 18 cycles.[144] If metastasis of the breast cancer to other organs has occurred, a loading dose of 4 mg/kg of TRZ is administered followed by maintenance doses of 2 mg/kg every 3 weeks for a total duration of one year.[145] Various studies have validated that TRZ can be used in combination with other chemotherapeutic agents such as paclitaxel, anthracycline based chemotherapies, as well as with cyclophosphamide.[39, 42] Several studies have cited that the combined administration of TRZ with anthracycline based chemotherapy is capable of reducing cancer related death by 33%.[39, 122, 144, 146, 147] Additionally, the use of TRZ in conjunction with a number of other chemotherapies such as Capecitabine, Cisplatin, Gemcitabine, and Vinorelbine has also been evaluated and shown to be effective.[148] Once administered, TRZ has a half-life of 28.5 days, which in comparison to other anthracycline based chemotherapies is quite long.[149] To date, the metabolic pathways for the elimination of TRZ remains undefined.[39, 121, 122, 150]

A number of multi-centred randomized clinical trials have evaluated the role of TRZ in the clinical setting. These studies include the Herceptin Adjuvant Trial (HERA), the National Surgical Adjuvant Breast and Bowel Project Trial B-31 (NSABP B-31), the North Central Cancer Treatment Group Trial N9831 (NCCTG-N-9831), and the Breast Cancer International Research Group 006 Data Trial (BCIRG 006).[113, 126, 146, 151-155] Patients in these trials received a cumulative dose of DOX or Epirubicin of 360 mg/m^2 and 720 mg/m^2, respectively.[113, 126, 146, 151-155] In

summary, these trials identified that after a minimum of four cycles of neo-adjuvant therapy followed by one year of adjuvant treatment with TRZ, the overall rate of recurrence cancer and mortality were reduced by 50% and 33%, respectively in women expressing HER2 positive breast cancer.[113, 126, 146, 151-155] TRZ, in addition to adjuvant anthracycline and taxane based chemotherapies are used as part of a routine treatment for HER2 positive breast cancers as they improve patient survival while reducing the risk of recurrence.

Clinical trials have also evaluated the efficacy of TRZ in conjunction with other anthracycline and taxane based chemotherapies against metastatic malignancies, which represent 10% of the breast cancer population.[39] Approximately 35% - 45% of metastatic breast cancer patients are HER2 positive.[39] Although palliative, chemotherapeutic agents including anthracyclines, Capecitabine, Docetaxel, Gemcitabine, Paclitaxel, and Vinorelbine, have been shown to improve the short term survival of metastatic breast cancer patients.[18, 156] The recent introduction of TRZ to the treatment regimen of HER2 positive breast cancer patients has significantly improved the survival outcome in this patient population.[39, 156] A multicentre clinical trial of 114 metastatic HER2 positive breast cancer patients demonstrated an overall response rate of 26% when TRZ monotherapy was used without prior use of an anthracycline based chemotherapy.[157] Overall, women with HER2 positive metastatic breast cancer experienced a 20% mortality risk reduction when they received both anthracycline and TRZ.[158] Specifically, a study by Stickeler *et al.* (2009) demonstrated that a total of 89% of HER2 positive metastatic breast cancer patients responded to TRZ therapy compared to only 39% of HER2 negative metastatic breast cancer patients who responded to the same therapy.[159] Not only do anthracycline based chemotherapies and TRZ significantly show greater responsiveness among HER2 positive patients individually, but in combination DOX+TRZ

decrease the risk of recurrence and progression by 33%.[39] Despite the beneficial anti-cancer properties of TRZ, when combined with anthracyclines, patients treated with this drug combination, are at a higher risk of developing chemotherapy induced cardiotoxicity.[39, 68, 91]

2.9 Cardiotoxicity: A Concise Definition

As stated by the American Society of Echocardiography's 2014 Guidelines for the multimodality imaging evaluation of patients during and after cancer therapy, Cancer Therapeutics-Related Cardiac Dysfunction (CTRCD) is defined as: a decrease in left ventricular ejection fraction (LVEF) of >10 percentage points to a value <53% (the normal reference value for 2D echocardiography).[160] Additionally, the observed decrease in LVEF must be confirmed by repeated cardiac imaging performed within 3 weeks of diagnosis of the initial decrease in LVEF.[160]

The Canadian Cardiovascular Society (CCS) published an updated set of Cardio-Oncology Guidelines in 2016, which provide recommendations for four key topics relating to CTRCD.[161] These areas include: i) identifying the high-risk population; ii) strategies for diagnosing and preventing CTRCD; iii) treatment of CTRCD; and iv) a multidisciplinary approach to the management of CTRCD.[161] The CCS Cardio-Oncology guidelines cite the multiple hit hypothesis as the framework for understanding CTRCD.[161] The multiple hit hypothesis suggests that patients who are at the highest risk of CTRCD are those who already possess traditional risk factors for atherosclerosis and heart disease, which are overwhelmed by the addition of cytotoxic cancer therapies.[161] Specifically, patients are considered high-risk if they possess one or more of the following comorbidities: LV systolic dysfunction, hypertension, myocardial ischemia/arterial thrombosis, or arrhythmias.[161] In contrast to the ASE 2014 Guidelines, the CCS Cardio-Oncology

2016 Guidelines cite 3D transthoracic echocardiography (TTE) as the modality of choice for the diagnosis of CTRCD. Virani *et al.* (2016) suggest 3D echocardiography as the preferred technique for the detection of cardiotoxicity as it is more accurate for the diagnosis of CTRCD and has better reproducibility than 2D echocardiography.[161] In addition to 3D echocardiography, a multigated acquisition (MUGA) scan and cardiac magnetic resonance (CMR) are also acceptable imaging modalities for the noninvasive diagnosis of CTRCD.[53, 161-163] Additionally, the New York Heart Association (NYHA) standards are used to classify the degree of chronic heart failure (CHF) from class I-IV based on the adverse side effects of adjuvant chemotherapy. The NYHA defines each class of CHF as follows: Class I) mild CHF with no limitation in physical activity; Class II) mild CHF with a slight limitation in physical activity; Class III) moderate CHF with marked limitation in physical activity that leads to CHF symptoms; and Class IV) severe shortness of breath at rest, with an inability to carry out physical activity without discomfort.[164]

The cardioprotective role of RAS antagonists, β-blockers, anti-oxidants, and statins have been investigated in a number of basic science and clinical studies for the management and prevention of CTRCD.[79, 165-173] We will further elaborate on the use of these medications in sections 2.16 – 2.19 of this thesis. As recommended by the CCS Cardio-Oncology 2016 Guidelines, there is a need for a multidisciplinary approach to the prevention, diagnosis, and treatment of cardiovascular disease in cancer patients.[161, 174] Currently, there are no benchmarks that have been established to help guide clinicians with regard to the timely access and assessment of patients who experience CTRCD. Additionally, there lacks a balance between patient wait times in the Cardio-Oncology clinic and the urgency of impending cancer treatments.[161] Therefore, Cardio-Oncology must be recognized as a distinct inter- and multidisciplinary patient-centered discipline, which requires the

collaboration of professionals across several medical fields in order to provide the best care to patients.

2.10 Doxorubicin-Induced Cardiotoxicity

The subspecialty of Cardio-Oncology was first introduced in 1967 with the recognition of Daunorubicin-induced cardiotoxicity. First produced as an improved form of Daunorubicin in 1969, DOX has become the most widely used broad-spectrum anti-tumor agent for its potent anti-cancer properties.[69, 175] DOX is highly effective in the cancer setting as it prolongs the survival of patients, but it's clinical use has been limited due to the adverse side effects associated with its administration.[69] The side effects associated with DOX treatment include nausea, vomiting, cardiac arrhythmia, and cardiac dysfunction.[91, 176] The most limiting side effect associated with DOX is its inherent cardiotoxicity, which is the pivotal reason for the development of Cardio-Oncology as a field of study.[177]

DOX is categorized as a Type I cardiac injury chemotherapeutic agent. [91, 178] Type I cardiac injury is defined by myocardial damage that results in the death of cardiomyocytes, typically incurred through the process of apoptosis.[91] This damage caused by anthracycline agents like DOX is irreversible and leads to adverse cardiac remodeling. Both the acute and chronic cardiotoxic effects of DOX-induced cardiotoxicity are observed through electrocardiographic T-wave inversions, damaged cardiac ganglia, myocyte death, and advanced CHF.[50-52, 176] These side effects are known to affect the long term health status of 8000 Canadian women annually.[179, 180] Additionally, Singal and Iliskovic (1998) demonstrated that a lifetime cumulative dose of DOX that exceeds 500 mg/m^2 of body surface area will place patients at an increased risk of developing cardiotoxicity.[91, 92, 103]

To elaborate, heart failure (HF) was shown to develop in 4% of cases where patients received a cumulative dose of DOX of 500-550 mg/m^2, 18% of patients who received a dose of 550-600 mg/m^2, and 36% or patients receiving a cumulative dose of 600 mg/m^2 or higher.[91, 181] These findings indicate that the degree of DOX-induced cardiotoxicity is proportional to the cumulative dose administered. Therefore, in an effort to minimize the damage inflicted upon the heart by DOX, patients can no longer receive a maximum lifetime cumulative dose that exceeds 500 mg/m^2.[1, 91, 92, 103] While 500 mg/m^2 is the maximum lifetime cumulative dose of DOX that can be administered to patients, treatment can be discontinued if Type 1 cardiotoxicity develops prior to achieving this dose. In these circumstances alternative treatment chemotherapeutic approaches are pursued.[178]

Additional risk factors associated with DOX-induced cardiomyopathy include an age greater than 70 years old, prior radiation therapy, as well as a past history of hypertension.[91, 182, 183] Typically, patients receiving high doses of chest radiation and DOX, as well as other chemotherapies with cardiotoxic side effects have an increased lifetime risk of cardiovascular disease (CVD).[177] This includes arrhythmias, pericardial disease, valvular heart disease, coronary artery disease, and heart failure.[177] Given the severity of the cardiotoxic side effects of DOX, patients should be assessed at the onset of their diagnoses for possible risk factors that may increase their susceptibility to cardiotoxicity.[184] Therefore, finding the optimal therapeutic window for each individual patient is essential to maintain the lowest possible degree of cardiotoxicity. Unfortunately, finding this equilibrium remains an ongoing challenge in today's clinical practice.

2.11 Trastuzumab-Induced Cardiotoxicity

Due to the potency of TRZ as an anti-cancer therapy, this monoclonal antibody has become part of the standard form of chemotherapy for the treatment of HER2 positive breast cancers. Despite the therapeutic benefits of TRZ, its use in treating women with HER2 positive breast cancer is often weighted against the risk of developing drug-induced cardiomyopathy, and as such is a significant concern. The cardiotoxic properties of TRZ are significantly potentiated when combined with standard anthracycline based chemotherapies.[50, 91, 92] It is known that 25% of women who receive anthracycline and TRZ chemotherapies are at risk of developing eventual cardiac dysfunction.[68, 91, 92, 183] Anti-cancer agents including TRZ can cause Type II cardiac injury, which is characterized by myocardial dysfunction.[178] As Type II cardiotoxicity does not cause adverse cardiovascular remodeling, it is generally considered to be reversible and is therefore dose independent.[160, 178, 185] Typically, ACE inhibitors and β-blockers are used to treat Type II cardiac dysfunction, which allows for a well-tolerated reintroduction of TRZ therapy.[186]

Three major clinical trials have evaluated the development of LV systolic dysfunction due to TRZ therapy in the adjuvant setting. In the HERA Trial, the incidence of symptomatic heart failure in women receiving TRZ was below 1%, while the NSABP B-31 Trial found this rate to be in 4.1% of HER2 positive breast cancer patients.[113, 125, 126, 146, 151, 152, 154] Similarly, the results from the NCCTG-N-9831 Trial placed the percentage of HER2 positive breast cancer patients who received TRZ therapy and developed CHF to be 3%.[113] Based on the results of these studies, the incidence of symptomatic heart failure in HER2 positive breast cancer patients treated with TRZ ranges between 1-4%. Additionally, Ewer and Lenihan (2008) performed a study to determine the prevalence and reversibility of TRZ-induced cardiotoxicity. The LVEF in 60% of the women who

developped TRZ-induced cardiotoxitity had recovered by the 6-month follw-up period when perscribed with the appropriate heart failure medications.[187-189] However, in real world practice outside of clinical trials that have stringent exclusion criteria and short follow-up periods, the extent to which TRZ mediated cardiotoxicity is reversible remains undefined.[189]

In order to evaluate a real word picture of DOX+TRZ mediated cardiotoxicity, our group evaluated 152 HER2 positive breast cancer patients who underwent adjuvant therapy with TRZ.[58] The study's objectives were to: i) evaluate the incidence of cardiac dysfunction; and ii) determine the reversibility of cardiotoxicity in a patient population with HER2 positive breast cancer.[58] This study revealed that TRZ-related cardiotoxicity developed in 24% of women with the majority of diagnoses being asymptomatic.[58] The study went on to describe cardiac risk factors that predisposed patients to developing TRZ mediated cardiotoxicity. Risk factors that contribute to the development of cardiotoxicity include: i) a family history of premature coronary artery disease (CAD); ii) a history of CVD; iii) pre-existing hypertension (HTN); iv) smoking; and v) age.[58] While previous clinical trials have shown that the incidence of TRZ mediated cardiotoxicity is less than 10%, our real-world study placed this risk at as high as 25%.[58] Additional real-world studies indicate that over 40% of women with HER2 positive breast cancers do not show any recovery despite the discontinuation of TRZ and treatment for heart failure.[58] The risk factors identified in this study are strongly recommended to be included into the screening criteria for women with HER2 positive breast cancers prior to the administration of TRZ for both prognostic and strategic treatment management.[58, 187, 188]

Women with metastatic breast cancer are at the greatest risk of developing cardiotoxicity as TRZ is administered in conjunction with other anthracycline based chemotherapies.[68] Studies have demonstrated that 27% of women in the metastatic setting who receive both TRZ and standard anthracycline chemotherapy develop NYHA Class III or IV heart failure.[125] Unfortunately, with no other alternatives, this aggressive treatment approach has become common practice for HER2 positive metastatic breast cancer patients. As a result, these women are expected to have an overall poor prognosis with a mean life expectancy of 9 to 12 months.[125]

2.12 Mechanisms of Chemotherapy-Induced Cardiotoxicity.

While there are multiple mechanisms in which DOX exhibits its anti-tumor activity, the mechanism of DOX-induced cardiac damage is non-specific.[51] To elaborate, DOX's anti-cancer capabilities include: DNA crosslinking, direct membrane damage, altered DNA binding, alkylation, histone eviction, free radical generation, and Topo-II inhibition. However, the myocyte cells within the myocardium are also adversely affected as these anti-tumor activities are not specific.[51, 92, 93, 95-97] Therefore, this universal mechanism results in cardiac damage via the apoptotic pathway, which ultimately leads to the development of heart failure.[91, 92, 179, 180]

In comparison, there are three main mechanisms that lead to TRZ induced cardiotoxicity. The first mechanism involves the activation of cardiomyocyte cell death through the initiation of the genetically programmed cell death pathway known as apoptosis.[115] The administration of TRZ alters the expression levels of pro- and anti- apoptotic proteins. Specifically, the expanding family of proteins known as the B-cell lymphoma 2 (Bcl-2) contain both pro- and anti- apoptotic elements.[115] A ratio between pro- and anti- apoptotic protein levels determines whether or not a

cell will initiate cell death or its survival pathway.[115] A study by Grazette *et al.* (2004) suggested that the binding of TRZ to HER2 receptors causes an immediate increase in the pro-apoptotic protein B-cell lymphoma extra-small (Bcl-X_S) levels, as well as a decrease in the anti-apoptotic protein B-cell lymphoma extra-large (Bcl-X_L) levels.[116] The Bcl-2 protein family plays a critical role in mitochondrial function, therefore an increased ratio of pro- to anti- apoptotic protein is beneficial towards fighting cancer but detrimental to cardiac health.[115] Increased levels of pro-apoptotic Bcl-X_S has been associated with mitochondrial dysfunction and has been known to initiate cell death in cardiomyocytes.[116, 190]

The second mode of action of TRZ induced cardiotoxicity is through the upregulation of RAS.[191] The myocardium is known to be stressed by the binding of TRZ to the HER2 receptors as this inhibits the cells survival pathways.[192] The increased stress levels cause the levels of circulating angiotensin-II (ANG-II) to increase significantly. In response to increased levels of ANG-II, a decrease in NRG-1 protein levels ensues, ultimately reducing the amount of a key regulator in the cell survival signaling pathways.[169, 193] Additionally, the activation of cellular survival pathways is reduced by ANG-II, which inhibits the already reduced amount of NRG-1 from binding to the HER4 receptor.[169, 193] Finally, ANG-II can bind the angiotensin-1 receptor (AT_1), which activates a cascade that leads to the production of ROS.[62, 194-197] Cyclic superoxide production of ROS potentiates TRZ induced cardiac dysfunction by increasing the levels of OS. [193, 198] The increase in OS levels negatively affects cardiomyocyte health and results in cardiac hypertrophy and heart failure.[193, 198] ANG-II induced OS activates members of the mitogen-activated protein kinase (MAPK) family, including apoptosis signal-regulating kinase (ASK1), c-Jun N-terminal kinase (JNK) and p38.[199-203] Activated JNK and p38 are associated with myocyte apoptosis and cardiac

pathologies that lead to the increased expression levels of pro-apoptotic genes [Bcl-2 associated X protein (Bax), Caspase-3, and poly (ADP-ribose) polymerase (PARP)] and decreased expression levels of anti-apoptotic genes (Bcl-X_L – as described above).[199-201, 203-206] Overall, this cascade leads to the induction of apoptosis and ultimately the heart failure that is diagnosed in women with HER2 positive breast cancer.[54-57, 60, 62, 65, 67-77, 195, 197, 199-204, 207]

Finally, the third mechanism of TRZ induced cardiac dysfunction is caused by the anti-dimerization properties of TRZ. The binding of TRZ to a HER2 receptor prevents it from dimerizing to another HER family receptor.[208, 209] By inhibiting this dimerization process cellular proliferation, angiogenesis, and cell survival pathways are downregulated, which ultimately causes decreased resilience of the cardiomyocytes.[110, 119, 137, 190, 209-211] Additionally, active cardiomyocytes have a high metabolic demand for adenosine triphosphate (ATP), which are produced by the mitochondria. Consequentially, this increased demand for ATP from the electron transport chain (ETC) in the mitochondria potentiates the production of ROS.[190, 193] With increased metabolic demands, cardiomyocytes place themselves at a higher risk of apoptosis through increased levels of OS and ROS.[212, 213] Cardiomyocytes are therefore unable to compensate for the excess stress, which leads to the development of cardiac dysfunction through the initiation of apoptotic cell death pathways.[190, 211-213]

2.13 Inflammation: A Response to Breast Cancer Therapy

The inflammatory response pathway is a defense mechanism of the body that can be activated by pathogen infiltration, infection, and/or tissue damage. The inflammatory pathway is an intricate cascade of signals that coordinates the communication between immune cells and blood vessels.

The inflammatory response is divided into four distinct phases: i) inflammatory inducers, the site of infection or tissue damage which initiates the response; ii) inflammatory sensors, where mast cells and macrophages are recruited to the site of infection or tissue damage; iii) inflammatory mediators, cytokines, and chemokines are activated in response to the infection or tissue damage; and iv) the tissues that have been affected respond.[214] There are three main transcription factors that act as key modulators of the inflammatory response pathway, which include nuclear factor kappa B (NF-κB), hypoxia-inducible factors-1 alpha (HIF-1α), and signal transducer and activator of transcription (STAT).[215-217] Porta *et al.* (2009) have demonstrated that chronic inflammation can lead to cancer through the continued activation of these transcription factors.[218]

NF-κB acts as a regulator of the acute phase of inflammation where homeostasis can be re-established after the induction of the inflammatory response pathway. NF-κB up-regulates downstream pro-inflammatory biomarkers including tumor necrosis factor-alpha (TNF-α), interferon-alpha (INF-α), interleukin 1-beta (IL-1β), and interleukin-6 (IL-6), which can lead to cardiac fibrosis and heart failure.[70-72, 215, 219] Additionally, TNF-α and IL-1β can activate NF-κB as they are a part of its feedback loop.[215] HIF-α plays a role in the cellular response to hypoxia. During the inflammatory response, immune cells involved in the response as well as in surrounding areas experience a decrease in the available oxygen levels.[216] As a defense mechanism of the body, HIF-α will activate the transcription of genes that control processes including angiogenesis and erythropoiesis when the level of circulating oxygen is too low in order to help these cells survive.[216] Several cytokines including IL-1β work alongside HIF-α to assist it in increasing its levels of transcription.[216] Additionally, the use of cytokines is mediated by NF-κB, which has been shown

to bind to the promoter regions of HIF-α and regulate transcription.[216] The STAT protein family serve multiple different functions within the inflammatory response pathway depending on the mediator of inflammation.[217] This mediator determines which cytokines are triggered to activate the STAT protein and will either upregulate or downregulate the inflammatory response.[217] For example, STAT1 and STAT2 are activated by IFNs when the body comes into contact with a viral infection.[217] In comparison, STAT6 is activated to help with the differentiation of helper T cells, which can positively influence allergic inflammation or negatively affect autoimmunity.[217]

As a general definition, inflammation is the result of a complex biological response of the body to harmful stimuli including pathogens, infections, and/or tissue damage, in order to provide protection. This process recruits immune cells, blood vessels, as well as several molecular mediators that include NF-κB, TNF-α, IL-1β, and IL-6, which eliminate the initial cause of cell injury, necrotic cells, and/or damaged tissue.[70-72] In doing so, these mediators initiate the repair process.[70-72] In 1863, Rudolf Virchow first hypothesized that cancer originated at the sites of chronic inflammation.[220] Presently, chronic inflammation contributes to 15-25% of all human carcinomas.[221-223] Recently, an increase in inflammation has been cited as a main contributor to the tumor microenvironment, which contributes to cellular proliferation, survival, and migration.[224] In accordance with this finding, Coussens *et al.* (2002) demonstrated that selectins, chemokines, and their respected receptors are recruited for the invasion, migration, and metastasis of cancers.[220] Specific inflammatory mediators that contribute to neoplasia by fostering a proliferative environment include: i) prostaglandins; ii) inflammatory cytokines such as IL-1β, IL-6, IL-15; TNF-α; and iii) chemokines including interleukin-8 (IL-8), and chemokine C-X-C motif ligand 1 (CXCL1).[221, 225] In addition to promoting tumor growth, inflammation also plays a role in

DNA damage as it produces ROS through various intracellular inflammatory mediators.[220, 221, 225] Leukocytes and other phagocytic cells contribute to DNA damage through the generation of ROS and reactive nitrogen species (RNS), which lead to both mutations and epigenetic alterations that may provide selective advantages during cellular proliferation.[220, 221, 226-229]

In addition to the aforementioned markers, polyunsaturated fatty acid (PUFA) derived oxylipins have recently gained traction as being key inflammatory mediators. Oxylipins are acquired either directly through the diet or from the elongation and desaturation of precursor PUFAs.[230] The most common and well-known oxylipins are eicosanoids which are derived from arachidonic acid (AA).[230] However, oxygenase enzymes are required in order for this process to take place. Specifically, the production of eicosanoids from AA requires cyclooxygenase (COX) enzymes. COX enzymes convert 20-carbon PUFAs into prostanoids (a type of eicosanoid), which have a characteristic five-carbon ring structure at the 8- and 12- carbon positions with at least one double bond.[230] Once produced, prostanoids are released into circulation where they bind to G-protein coupled receptors on the cell surface to exert their effect.[230] COX-derived prostanoids are involved in a number of different processes including modulating the inflammatory response.[230, 231]

In recent years, a larger focus has recently been placed on investigating the inflammatory and OS mechanisms related to DOX+TRZ mediated cardiotoxicity.[54-57, 60, 62, 65, 67-77] Specifically, the study conducted by Bozkurt *et al.* (1998) showed that the administration of anthracycline based chemotherapeutic agents such as DOX was associated with increased inflammation within the myocardium and vasculature.[71] This study later went on to describe that the increase in inflammation can be measured by the expression of tumor necrosis factor-alpha (TNF-α).[71]

Overall, as DOX+TRZ mediated cardiotoxicity is multifactorial, including the up-regulation of pro-inflammatory mediators and increased OS production, further research is required to mitigate the adverse side effects of these common anti-cancer drugs (Figure 9). Specifically, this study will elucidate the mechanistic role of inflammation in DOX+TRZ mediated cardiotoxicity as well as investigate whether the anti-inflammatory agent flaxseed may prevent this adverse side effect from occurring.

2.14 Oxidative Stress: A Response to Breast Cancer Therapy

Activated oxygen species are produced by the incomplete reduction of oxygen, which results in singlet oxygen molecules, superoxide radicals, and hydroxide radicals.[195] ROS are detrimental to surrounding tissues as they have short half-lives, an unstable nature, and are highly reactive.[232] Additionally, ROS react with PUFAs, which initiate a cascade of reactions that result in lipid peroxidation.[195] Free radicals are capable of damaging double-stranded DNA as well as oxidizing sulfhydryl (thiol/-SH) groups found within proteins.[233] Furthermore, cardiac dysfunction has been noted as the end product of OS induced auto-oxidation of catecholamines.[70, 195] Similarly, ischemia-reperfusion studies have shown that abnormal cardiomyocyte function is a result of ROS interfering with the calcium transport in the sarcoplasmic reticulum.[195] Ultimately, ROS production can have devastating effects on physiology that can lead to disease. Due to the increased awareness of the potentially damaging effects of ROS, the investigation into the mechanisms of how chemotherapeutic regimens increase ROS have been of growing interest.

In general terms, OS is the result of an imbalance between the production of ROS in a biological system and its ability to neutralize these reactive intermediates. An increase in OS has been cited

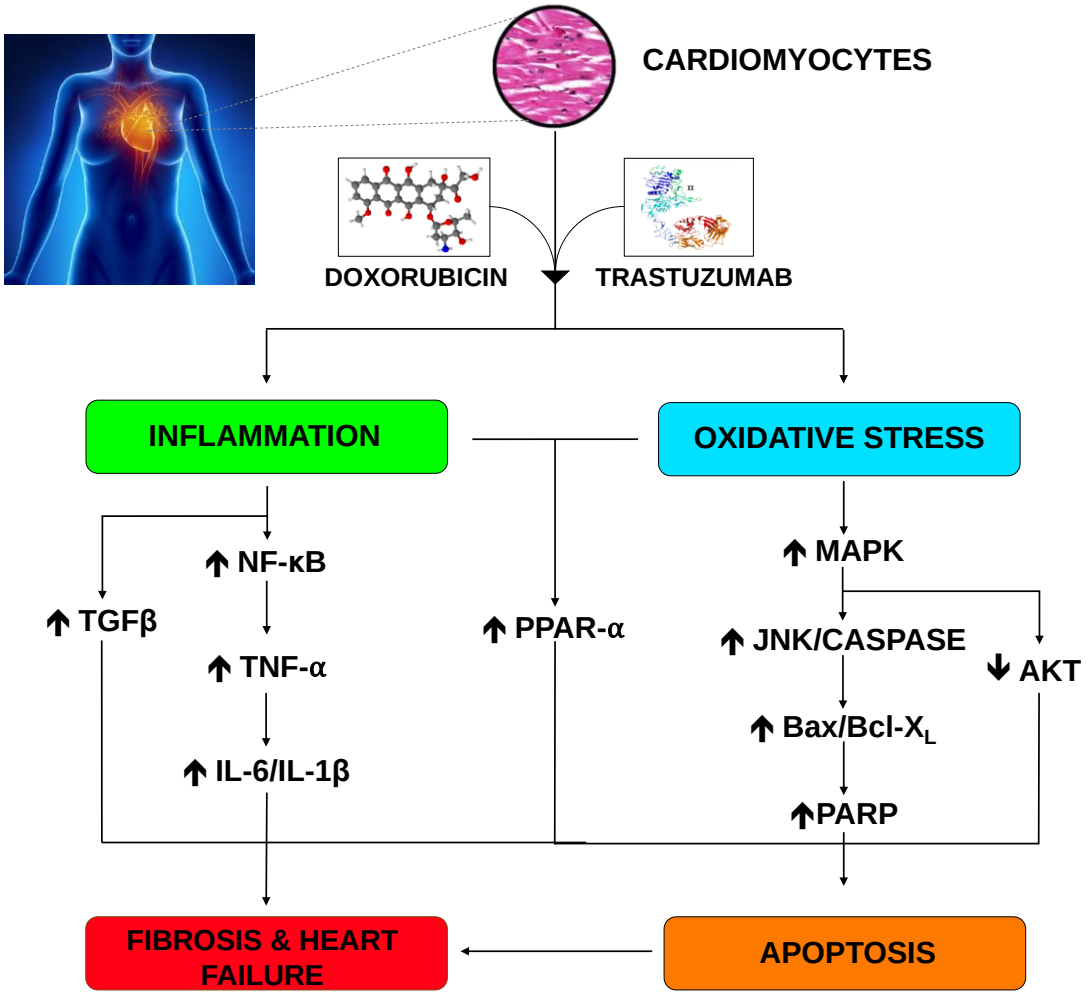
as one of the main contributors to chemotherapy related cardiac dysfunction, especially in the breast cancer setting when anthracycline based agents and monoclonal antibodies are used as first line treatment.[195] Anthracycline based agents are known to influence two specific signaling pathways: mitogen-activated protein kinases (MAPKs) and the peroxisome proliferator-activated receptor (PPAR) family.[73, 75, 234]

Breast cancer therapy has a significant impact on mitogen-activated protein kinases (MAPK), which result in increased levels of OS. MAPKs act in a signaling cascade that relay signals intracellularly from the cell surface to the nucleus.[234] Several cellular processes including cell proliferation, differentiation, and apoptosis, are directly influenced by the MAPK signaling pathways.[234] MAPK dependent pathways like JNK and p38 are upregulated by chemotherapeutic agents used in breast cancer, which leads to increases in the expression levels of pro-apoptotic proteins including Bax, Caspase-3, and PARP, while decreasing the expression levels of anti-apoptotic proteins like Bcl-X_L.[199-201, 203, 204] Additionally, a decrease in the expression levels of protein kinase B (AKT) due to anthracycline based chemotherapy leads to the downregulation of cell survival in cardiomyocytes as well as increased levels of OS. [235, 236] AKT has been shown to promote cellular survival through the phosphorylation of its substrates, which in turns affects the native function of the apoptotic machinery.[235, 236] The clinical use of chemotherapy regimens for women with breast cancer have been significantly impacted by the effects that increased levels of OS have on the MAPK pathway and cardiomyocytes.

DOX related increases in OS also up-regulates the peroxisome proliferator-activated receptor (PPAR) family.[73] PPARs are ligand-activated transcription factors that belong to the superfamily

of nuclear receptors.[73, 237] These subcellular organelles are involved in many metabolic processes including inflammation, fatty acid β-oxidation, and cholesterol metabolism. [238-241] PPAR is naturally activated into a heterodimer state by lipid derived substrates in association with a co-activator complex of peroxisome proliferators response elements (PPREs).[237] These PPREs function by binding to DNA sequences at the promoter region to regulate gene expression.[237] There are three subtypes of PPAR: PPAR-α, PPAR-γ, and PPAR-δ, which are encoded by separate genes and expressed in many species including rodents and humans.[242, 243] Of these three subtypes, PPAR-α is abundantly expressed in tissues with high levels of fatty acid β-oxidation including the heart, liver, kidney, pancreas, and skeletal muscle.[73, 244, 245] Normally, heart tissue utilizes fatty acid β-oxidation as the major source of energy. [73, 240, 246, 247] However, with an increased demand from the heart due to the toxic effects of anthracycline based chemotherapies, higher levels of oxygen are consumed. As a result of this increase in oxygen demand, an imbalance in the production and neutralization of ROS occurs.[73, 240, 246, 247] As a compensatory mechanism of the body to the increase in fatty acid β-oxidation the levels of PPAR-α protein are increased.[75] This increase in PPAR-α protein predisposes the heart to contractile dysfunction, which eventually results in DOX mediated cardiomyopathy (Figure 9).[75] Specifically, this study will elucidate the mechanistic role of OS in DOX+TRZ mediated cardiotoxicity as well as investigate whether flaxseed, in its role as an anti-oxidant may prevent this adverse side effect from occurring.

Figure 9: The pathogenesis of DOX+TRZ mediated cardiotoxicity.



In experimental models of cardiac injury due to DOX+TRZ, pro-inflammatory markers including NF-κB, TNF-α, PPAR-α, and IL-6 /IL-1β are up-regulated leading to cardiac fibrosis (increased TGFβ) and heart failure. Additionally, DOX+TRZ mediated OS leads to activation of PPAR-α, MAPK, JNK/Caspase, Bax/Bcl-X_L, PARP leading to increased apoptosis and cardiotoxicity. Bax, Bcl-2 associated X protein; Bcl-X_L, B-cell lymphoma extra-large; DOX, Doxorubicin; IL-1β, interleukin-1 beta; IL-6, interleukin-6; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; OS, Oxidative Stress; PARP, poly (ADP-ribose) polymerase; PPAR-α, peroxisome proliferator-activated receptor-alpha; TGFβ, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; TRZ, Trastuzumab.

2.15 Prevention of Chemotherapy-Induced Cardiotoxicity

Recent advancements in the understanding of chemotherapy induced cardiotoxicity as well as novel echocardiographic imaging techniques has allowed for the early detection of LV systolic dysfunction in cancer patients. The focus has now been shifted to early intervention and prevention of chemotherapy induced cardiac dysfunction.[56, 57, 167, 248-250] By implementing protocols to protect the cardiovascular health of patients during anti-cancer therapy, we can dramatically improve a patient's quality of life and overall chances of survival.[251, 252] One of the key strategies for preventing chemotherapy induced cardiac dysfunction is to focus on treating the patients with the highest risk of developing heart failure from their anti-cancer therapy regimen.[167, 253, 254] Individuals at an increased risk of developing chemotherapy induced cardiotoxicity include those with comorbidities including: i) other cardiovascular (CV) disorders including hypertension (HTN) and CAD ii) diabetes; iii) cardiovascular risk factors including smoking, obesity, use of alcohol, sedentary lifestyle, and diet; iv) advanced age; and v) exposure to ionizing radiation and cardio-toxins.[161, 253-255] Current intervention strategies for these patients involve the adjustment of chemotherapy drug dosages, prescription and substitution of a less cardiotoxic agent, as well as the use of cardioprotective medications.[253, 254]

To date, the Canadian health care system still lacks consensus and guidelines for the prophylactic administration of cardioprotective therapies as primary prevention against chemotherapy mediated cardiotoxicity.[161, 167, 254, 256] However, the CCS recently suggested the use of the following medications for cancer patients who are at a high risk of developing cardiovascular complications following DOX+TRZ anti-cancer therapy: i) angiotensin receptor blockers (ARB); ii) ACE inhibitors; iii) β-blockers; and/or iv) statins.[161] In response to the growing need for cardioprotective

agents, several clinical trials have shifted their attention to the prevention of cardiac damage in breast cancer, sarcoma, lymphoma, and leukemia patients who are on anthracycline-based chemotherapy regimens.[161, 167] Unfortunately, results from these studies have been inconclusive or contradictory due to several limitations including small sample size, variable results, and/or early termination of the study.[167] Currently, there is little evidence on the prophylactic use of heart failure medications in the prevention of chemotherapy related cardiotoxicity in a variety of cancer settings.[161, 167]

Potential cardioprotective agents have been selected based on the underlying mechanisms of chemotherapy induced cardiotoxicity.[165, 257] Some cardioprotective agents were investigated based on their capability to decrease the workload on the heart, while other agents may directly affect the heart at a molecular level by reducing the levels of OS, mitochondrial damage, and/or apoptosis of the cardiomyocytes.[165, 257] In the setting of chemotherapy induced cardiac dysfunction, the cardioprotective role of RAS antagonists, β-blockers, anti-oxidants, and statins have been investigated in a number of basic science and clinical studies.[66, 79, 165-173]

2.16 Prevention of Chemotherapy-Induced Cardiotoxicity: RAS Antagonists

RAS antagonists are a group of blood pressure lowering medications, which include: direct renin inhibitors (DRI) including Aliskiren; ACE inhibitors such as Perindopril and Enalapril; as well as ARB's including Valsartan and Candesartan.[66, 257-259] Aliskiren can benefit both the cardiovascular and renal systems, while ACE inhibitors decrease the risk of myocardial infarction, stroke, and death in high risk patients without a diagnosed form of cardiomyopathy.[260-263] Patients with CHF are recommended to start treatment with a Class I medication such as the ACE inhibitor

Perindopril, which is administered after the development of LV systolic dysfunction.[252, 264-268] However, it has yet to be determined whether ACE inhibitors like Perindopril can be used prophylactically to prevent adverse cardiovascular remodeling.[161, 167, 254, 256] Based on a number of clinical trials, ARBs including Valsartan have been shown to decrease the morbidity and mortality associated with cardiomyopathy.[269, 270] Additionally, the potential for RAS antagonists to exert anti-inflammatory, anti-oxidant, anti-apoptotic, and anti-fibrotic properties have been demonstrated by several studies.[261, 262, 271-277]

There have been a number of basic science and clinical trials that have investigated the potential cardioprotective role of RAS antagonists in the prevention of chemotherapy induced cardiac dysfunction.[61, 278] The study by Akolkar *et al.* (2015) establish a chronic *in vivo* murine model of DOX and/or TRZ mediated cardiotoxicity where mice were prophylactically administered either placebo, Aliskiren (DRI), Perindopril (ACE inhibitor), or Valsartan (ARB) for a total of 13 weeks.[61] Increased LV cavity dimensions and reduced LVEF were observed in mice receiving the DOX only treatment or the combination treatment with DOX+TRZ.[61] However, the prophylactic administration of RAS antagonists partially prevented adverse cardiovascular remodeling in these groups.[61] In another study conducted by Abd El-Aziz *et al.* (2001), the ACE inhibitors Captopril and Enalapril were effective at preventing DOX induced cardiotoxicity by maintaining the balance between ROS production and the anti-oxidant reserve.[279] Additionally, Captopril and Enalapril were able to maintain respiratory efficiency of the mitochondria.[279]

Several clinical trials have evaluated the potential cardioprotective role of RAS antagonists against chemotherapy induced cardiac dysfunction.[78, 79, 170] In the recent PRADA Trial, Gulati *et al.* (2016)

evaluated the use Candesartan, an ARB as well as the β-blocker Metoprolol as a potential cardioprotective agent in women with breast cancer receiving an anthracycline-based chemotherapy either with or without TRZ.[79] In this study, cardiovascular remodeling was measure by changes in LVEF that were obtained from CMR.[79] Although prophylactic treatment with Candesartan partially preserved LVEF, there were no cardioprotective effects observed in the Metoprolol treatment arm.[79] In comparison, the opposite was found to be true in the MANTICORE Trial.[78] Pituskin *et al.* (2017) evaluated the potential cardioprotective role of the ACE inhibitor Perindopril versus that of the β-blocker Bisoprolol in women with breast cancer who were being treated with the monoclonal antibody TRZ.[78] In this study, cardiovascular remodeling was measure by changes in LV end diastolic volume as well as LVEF using CMR.[78] After a mean follow-up of nearly 1 year, it was found that the β-blocker Bisoprolol attenuated the decrease in LVEF as compared to Perindopril and placebo groups.[78] However, in a study performed by Janbabi *et al.* (2017), it was observed that treatment with the ACE inhibitor Enalapril was effective in preserving both the systolic and diastolic function of the heart in a population of cancer patients who received an anthracycline-based chemotherapy.[170] Despite several clinical trials investigating the role of RAS antagonists in the prevention of chemotherapy induced cardiotoxicity, a lack of global consensus remains.

2.17 Prevention of Chemotherapy-Induced Cardiotoxicity: β-Blockers

Beta-adrenergic blocking agents including β-blockers, are a class of medications that reduce a patient's blood pressure.[264] β-blockers function by blocking the effects of the hormone epinephrine, also commonly known as adrenaline. Once administered, a β-blocker will slow the

heart rate (HR) to reduce the overall force or workload that is exerted by the heart thereby reducing the patient's blood pressure. β-blockers are used for the treatment of cardiovascular diseases including HTN, LV systolic dysfunction, valvular heart disease, and arrhythmias.[257, 264, 280] However, β-blockers also have potentially potent anti-cancer, anti-oxidant, and anti-apoptotic properties.[165, 257, 280-282]

Several clinical trials have investigated whether β-blockers can exert cardioprotective effects against chemotherapy induced cardiomyopathy.[282, 283] Kalay *et al.* (2006) conducted a small randomized, placebo-controlled study, which investigated the potential cardioprotective role of the β-blocker Carvedilol (12.5 mg/day) in patients receiving anthracycline-based chemotherapies for their underlying malignancy.[282] At the conclusion of the 6-month study, it was determined that Carvedilol preserved LV function and prevented anthracycline mediated cardiomyopathy.[282] Similarly, the OVERCOME Trial by Bosch *et al.* (2013) assessed the potential cardioprotective properties of Carvedilol and the ACE inhibitor Enalapril in the prevention of chemotherapy induced LV systolic dysfunction in patients with hematological malignancies.[80] Bosch *et al.* (2013) concluded that both Carvedilol and Enalapril were cardioprotective.[80] Contradictory, the recent CECCY Trial led by Avila *et al.* (2018) determined that Carvedilol had no impact on the incidence of early onset LVEF reduction among patients with HER2 negative breast cancer undergoing anthracycline based chemotherapy.[284] However, this prospective, randomized, double-blinded, placebo-controlled study confirmed that Carvedilol did result in a significant reduction in troponin levels as well as diastolic dysfunction.[284] As previously discussed, the results from the recent MANTICORE Trial by Pituskin *et al.* (2017) determined that the β-blocker Bisoprolol

demonstrated cardioprotective properties against LV remodeling in breast cancer patients who received TRZ, while the ACE inhibitor Perindopril did not exert any cardioprotective properties.[78] However, the aforementioned PRADA Trial displayed contradictory results in that the ARB Candesartan exhibited cardioprotective effects while the β-blocker Metoprolol did not.[79] It must be noted that adverse LV remodeling associated with chemotherapy was not prevented by the agents in either trial.[78, 79] Despite several clinical trials investigating the role of β-blockers in the prevention of chemotherapy induced cardiotoxicity, a lack of global consensus remains.

2.18 Prevention of Chemotherapy-Induced Cardiotoxicity: Anti-Oxidants

ROS production is a fundamental mechanism of DOX induced cardiotoxicity.[195] The synthesis of ROS is associated with the MAPK and PPAR signaling pathways, which directly influence several processes including cellular proliferation, differentiation, replication via mitosis, apoptosis, inflammation, fatty acid β-oxidation, and cholesterol metabolism.[73, 75, 195, 233, 234, 238-241] Within the past two decades, several basic science and clinical studies have evaluated the use of anti-oxidants in the prevention of chemotherapy induced cardiomyopathy due to their capability to neutralize ROS and maintain a homeostatic balance within the body. These studies investigated anti-oxidants including Probucol, Vitamin C, superoxide dismutase (SOD), N-acetylcysteine (NAC), N-acetylcysteine amide (NACA), and glutathione catalase.[64, 95, 285-289]

Probucol is an effective lipid-lowering compound which is used clinically to decrease both serum low-density lipoproteins (LDL) and high-density lipoproteins (HDL), as well as to both prevent and treat atherosclerosis.[69, 290, 291] Additionally, Probucol has been shown to possess potent anti-

inflammatory and anti-oxidative properties with its anti-oxidant ingredient being bisphenol, an organic compound with two hydroxyphenol functionalities.[290, 292] In 1997, Iliskovic and Singal compared the efficacy of the cardioprotective properties of Probucol against Lovastatin (lipid-lowering medication) against anthracycline based chemotherapy.[293] This study determined that in addition to the inability of Lovastatin to lower the levels of plasma triglycerides and HDL in the DOX treated group, Lovastatin only partially prevented DOX induced changes in blood pressure, ascites, and mortality as compared to the Probucol treated group.[293] In addition to the lipid-lowering properties of Probucol, this drug also exerts a protective effect on the function of vascular endothelial cells by driving nitric oxide (NO) and nitric oxide synthase (NOS) production too enhance endothelial function and NO-mediated relaxation.[294]

In a study by Siveski-Iliskovic *et al.* (1994) that was later replicated by Kumar *et al.* (2001), Probucol was shown to protect against DOX mediated cardiomyopathy in rats. [295-300] Probucol exerted its' cardioprotective effects by maintaining the anti-oxidant reserve, downregulating apoptosis of the cardiomyocytes, and by preventing detrimental hemodynamic changes.[295-300] More recently, a basic science study performed by Walker *et al.* (2011) explored whether or not Probucol could inhibit DOX+TRZ induced cardiotoxicity by reducing the levels of OS in an acute murine model of chemotherapy induced cardiac dysfunction.[54] Prophylactic administration of Probucol lessened the degeneration of myofibrils and decreased cardiac apoptosis.[54] Although Probucol has demonstrated itself as an effective prophylactic agent in the *in vivo* settings of chemotherapy induced cardiomyopathy, its safety is yet to be investigated in clinical trials.[54]

The cysteine derived pharmaceutical agent N-acetylcysteine (NAC), has been used clinically for the treatment of acetaminophen toxicity, mucolytic therapy, and as a nephron-protective agent against radio-contrast induced nephropathy.[61, 287, 288, 300-309] The potential cardioprotective effect of NAC is drawn from its structure, which contains L-cysteine, a precursor of the biological anti-oxidant glutathione (GSH).[310] Therefore, NAC is capable of dissipating any increases in OS by replenishing the GSH reservoir and neutralizing ROS.[287] Unverferth *et al*. (1983) evaluated the potential cardioprotective role of NAC against DOX mediated cardiotoxicity in the clinical setting.[311] A total of 20 patients were randomized to either placebo or NAC group where patients received prophylactic admiistraion of NAC (140 mg/kg) one hour prior to their treatment with DOX.[311] Endomyocardial biposies were taken at three time points: baseline, 4 hours after NAC administration, and 24 hours after NAC administration.[311] Unfortunately, this trial failed to show any beneficial effects of NAC against DOX mediated cardiomyopathy as no signifigant differences with respect to cardiomyocyte injury were observed.[304, 311]

Recent efforts to prevent chemotherapy mediated cardiomyopathy in the evolving field of Cardio-Oncology led to the creation of a thiol structural analog of NAC known as N-acetylcysteine amide (NACA). Additionally, the carboxylic acid functional group (-COOH) which makes up part of the chemical structure of NAC has been replaced with an amide functional group ($-NH_2$) to form NACA.[300, 306] The addition of the $-NH_2$ functional group significantly increases the bioavailability of NACA.[300, 306] The improved chemical structure of NACA is what generates its potent anti-oxidative properties when compared to NAC.[312] NACA maintains a homeostatic balance between the production and neutralization of ROS through the maintenance of cellular GSH levels, which is facilitated by the transfer of its thiol group (-SH) to oxidized glutathione (GSSG).[300] NACA can

be administered at lower dosages due to its increased bioavailability and potency.[288, 300, 306, 307] This reduces the number of adverse side effects associated with NAC toxicity, which makes NACA a prime candidate for its use in the clinic.[288, 300, 306, 307] NACA is capable of scavenging free radicals.[300, 306, 307] NACA protects the blood brain barrier of mice from OS due to methamphetamine (250 mg/kg) exposure, and is capable of protecting erythrocytes by inhibiting matrix metalloproteinases, the JNK pathway, and the MAPK pathway.[300, 306, 307] Additionally, *in vitro* and *in vivo* studies have investigated the potential cardioprotective role of NACA against anthracycline based chemotherapy induced cardiomyopathy.[64, 288] A study performed by Goyal *et al.* (2016) investigated whether the anti-oxidant NACA could inhibit DOX+TRZ induced cardiotoxicity in an acute *in vivo* female mouse model of chemotherapy induced cardiac dysfunction.[64] This group determined that the prophylactic administration of NACA was able to preserve systolic function and attenuate the cardiotoxic effects of DOX+TRZ chemotherapy.[64] To date, no clinical studies have investigated the role of NACA in the prevention of chemotherapy induced cardiac dysfunction.

2.19 Prevention of Chemotherapy-Induced Cardiotoxicity: Statins

A class of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors also commonly known as stains, function by blocking cholesterol systhesis.[313, 314] Statins are typically used to treat patients with hyperlipidemia but may also be considers a first or second line preventative strategy for patients with a high risk for a myocardial infarction (MI) and/or stroke.[280] Statins have been shown to have beneficial anti-oxidative, anti-inflammatory, immunomodulatory, and cardioprotective properties, the later especially in the cancer setting.[165, 166, 168, 171, 172, 315, 316] A study by Raid *et al.* (2009) showed that pre-treatment with Fluvastatin improved the systolic

function of mice that developed DOX induced cardiomyopathy.[166, 171] Additionally, the expression levels of the anti-oxidant superoxide dismutase 2 (SOD2) were upregulated, while cardiac nitrotyrosine, pro-apoptotic, and pro-inflammatory markers were downregulated by Fluvastatin.[166, 171] In a separate study by Ramanjaneyulu *et al.* (2013), mice were prophylactically treated with Atorvastatin 1 hour prior to the administration of DOX.[314] The mice in the Atorvastatin treated group demonstrated a decrease in OS as well as reduced levels of cellular damage within the myocardium.[314]

In the clinical setting, Acar *et al*. (2011) evaluated the potential cardioprotective role of Atorvastatin in patients who were treated with an anthracycline based chemotherapy.[259, 317] In this study, patients were randomized to either receive daily prophylactic treatment of: i) Atorvastatin (40 mg) prior to the administration of their chemotherapy; or ii) chemotherapy alone.[259, 317] LVEF values remained unchanged after a 6-month follow up in the Atorvastatin group while the control group demonstrated a significant decreased in LVEF by this time.[259, 317] In addition to the echocardiographic parameters measured in this study, C-reactive protein levels were calculated and determined to be lower in the Atorvastatin treated group as compared to control.[259, 317] Similarly, Chotenimitkhun *et al.* (2015) demonstrated that treatment with statins prevented a decline in LVEF in patients receiving treatment with an anthracycline based chemotherapy.[318] In a separate observational study, Seicean *et al.* (2012) followed patients who had been diagnosed with breast cancer for 2.4 years and discovered that patients who were administered statins during their treatment period with an anthracycline based chemotherapy had a lower incidence of heart failure and cardiac-related mortality than those women who were not on statins.[168] In a recent study by Calvillo-Argüelles *et al.* (2019), the cardioprotective role of statins were evaluated in patients

with HER2 positive breast cancer that were receiving treatment with TRZ.[319] This study included a total of 129 patients aged 62±9 years.[319] A total of 43 patients were placed on statin therapy for the duration of their cancer treatment, whose median TRZ exposure time was 11.8 months.[319] When compared to the control patients, the patients who were treated with statins had a higher incidence of diabetes ($p<0.001$), hypertension ($p<0.001$), and coronary artery disease ($p=0.04$).[319] After an 11 months follow-up period, a significant change in LVEF was observed in the control group (median -6%, IQR -10% to -1%; $p< 0.001$) but not in the statin group (median 0%, IQR -5% to +3%; $p=0.27$).[319] Furthermore, after the analyses were adjusted, statin treatment was found to be independently associated with a lower risk of cardiotoxicity with an odds ratio of 0.32 and 95% confidence interval of 0.10-0.99 ($p=0.049$).[319]

Although pharmacotherapies including RAS antagonists, β-blockers, anti-oxidants, and statins are commonly used after the development of systolic dysfunction, little is known on whether alternative approaches including nutraceuticals agents like flaxseed can be used at the onset of treatment to prevent chemotherapy induced cardiotoxicity. As approximately 30% of breast cancer patients already consume alternative medicine supplements including FLX, an important opportunity exists to evaluate the potential cardioprotective role of this nutraceutical agent.[83]

2.20 Flaxseed: A History Lesson

Flaxseed, *Linum usitatissimum*, is a whole grain commodity that dates back to the Upper Paleolithic (Old Stone Age), which took place over 30,000 years ago.[320-322] FLX is an annual plant that can grow to be up to 1.2 meters tall.[323] The plant itself is a tall slender stem with glaucous green coloured lanceolate leaves (20 – 40 mm in length, 3 mm in width).[323] The stem blooms into

a pure, pale blue, 5 petaled flower that is 15 – 25 mm in dimeter.[323] The fruit produced is a round, dry capsule with a 5 – 9 mm diameter, which contains several of the flaxseeds (4 – 7 mm in length).[323]

The earliest evidence of human use of FLX came from the present-day Republic of Georgia, where FLX was found to be used as a textile.[321, 322, 324] Specifically, wild FLX fibers were spun, dyed, and knotted into linen.[321, 322, 324] FLX was extensively cultivated in ancient Egypt to produce linen that was used to entomb mummies.[325] Additionally, Egyptian priests wore only linen as it was considered a symbol of purity.[326] Soon after, FLX-based linens were traded throughout the Mediterranean and the Roman Empire as they were used to make the sails of ships.[327] As the Roman Empire fell, the production of FLX decreased until it was revived by Charlemagne, the Holy Roman Emperor of the 8th century.[326] Eventually, Flanders, Belgium became the major producer for industrial linen in the European Middle Ages.[326] The use of the FLX crop continued to spread across European and Asian countries including Switzerland, Germany, China, and India over 5,000 years ago.[328, 329] Eventually, FLX was brought over to North America by colonists where it initially flourished. However, FLX crops quickly became obsolete by the early 20th century as they were replaced with cotton.[330] Subsequently, FLX lost its importance as a commercial crop as the availability of more durable fibers became more easily accessible.[330] However, in present day society, FLX is primarily cultivated for human consumption.

In 2014, Canada produced approximately 1/3 of the global total of FLX (2.65 million tons).[331] Within Canada, Manitoba is the leading producer of FLX contributing an impressive 12% to the worldwide supply.[331] In 2016, the top five major producers of FLX include: i) Russia; ii) Canada;

iii) Kazakhstan; iv) China; and v) the United States.[331] This revival in the production of FLX crops is primarily attributed to its nutritional and health benefits.

2.21 The Nutritional Value of Flaxseed

The Reference Daily Intake (RDI) is the daily intake level of dietary nutrients like protein, fiber, or carbohydrates, that is considered sufficient to meet the requirements of 97 – 98% of healthy individuals.[332] For example, the FDA has recommended that individuals should consume a daily value of the following macronutrients: total fat (78 g), saturated fatty acids (20 g), cholesterol (300 mg), sodium (2300 mg), potassium (4700 mg), carbohydrates (275 g), dietary fiber (28 g), and protein (50 g).[332, 333] Any foods that exceed 19% of the daily values listed above, are considered to contain high levels of that nutrient. Based on this definition, FLX is considered to contain high levels (>19% daily value) of protein, dietary fiber, several B vitamins, and dietary minerals.[334] Specifically, the nutritional value within a 100 g serving of FLX is summarized in Table 1 below.[335, 336]

Table 1: Nutritional value for a 100 g serving of flaxseed. [335, 336]

Nutritional Value per 100 g (3.5 oz)		
Energy	2,234 kJ	(534 kcal)
Carbohydrates	28.88 g	
Sugars	1.55 g	
Dietary fiber	27.3 g	
Fat	42.16 g	
Saturated	3.663 g	
Monounsaturated	7.527 g	
Polyunsaturated	28.730 g	
omega-3	22.8 g	
omega-6	5.9 g	
Protein	18.29 g	
Vitamins	**Quantity**	**%DV**
Thiamine (B1)	1.644 mg	143%
Riboflavin (B2)	0.161 mg	13%
Niacin (B3)	3.08 mg	21%
Pantothenic acid (B5)	0.985 mg	20%
Vitamin B6	0.473 mg	36%
Vitamin C	0.6 mg	1%
Minerals	**Quantity**	**%DV**
Calcium	255 mg	26%
Iron	5.73 mg	44%
Magnesium	392 mg	110%
Phosphorus	642 mg	92%
Potassium	813 mg	17%
Zinc	4.34 mg	46%

Recently, there has been an increasing public awareness of the importance of consuming FLX. This is facilitated by the ease of use as well as the increased public awareness of the health benefits that are associated with regular consumption of FLX. FLX is a non-toxic whole grain composed of high concentrations of health promoting components including omega-3 fatty acids and plant lignans, which have potent anti-inflammatory, anti-oxidative, and anti-fibrotic properties. [84, 337-339]

2.22 The Health Benefits of Flaxseed

FLX has been shown to possess an abundance of the anti-inflammatory omega-3 fatty acid: ALA.[84, 337-339] The potent anti-inflammatory activity of ALA stems from its capability to decrease the expression of several inflammatory biomarkers including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and peroxisome proliferator activated receptor alpha (PPAR-α).[84, 337-339] The downregulation of these biomarkers has led to several cardioprotective effects of ALA including: arrhythmia prevention, lipid lowering properties, vascular relaxation improvement, anti-inflammatory responses, anti-atherosclerotic effects, and weight management.[65, 69, 70, 76, 77, 190, 340-351]

Specifically, Mozaffarian *et al.* (2005) performed a meta-analysis of randomized controlled trials that evaluated the role of high-ALA content oils on HR.[341] The investigators concluded that high-ALA content oils significantly reduce HR with the greatest effect being in individuals who presented with a higher baseline HR and who received treatment for a longer duration.[341] An additional study by Leaf *et al.* (2003) concluded that n-3 polyunsaturated fatty acids (including ALA) may be used as a form of clinical prevention for sudden cardiac death.[343] In addition to its anti-arrhythmia effects, ALA has been cited by multiple studies for its potent lipid-lowering

capabilities. A meta-analysis of these studies was performed by Pan *et al.* (2009) who summarized that FLX significantly reduced circulating total and LDL-cholesterol levels, but that these changes were dependent on sex, baseline lipid profiles, and the type of intervention.[340]

FLX has also shown to improve vascular relaxation. Specifically, Rodriguez-Leyva *et al.* (2013) performed the FlaxPAD clinical trial, which evaluated the effect of FLX on the systolic and diastolic blood preassures of patients with peripheral artery disease (PAD).[346] This prospective, double-blinded, placebo-controlled, randomized clinical trial included a patient population of n=110 who consumed a daily total of 30 g of milled FLX for 6 months.[346] Overall, there was a 10 mmHg and 7 mmHg decrease in systolic and diastolic blood pressures, respectively in this patient population.[346] These findings characterized FLX as having one of the most potent anti-hypertensive effects ever achieved by a dietary intervention.[346] Following the success of this clinical trial, Caligiuri *et al.* (2014) performed a sub-analysis on the FLaxPAD population to elucidate the anti-hypertensive mechanisms of FLX.[345] This study concluded that FLX inhibited soluable epoxide hydrolase, which is a pharmacological target for anti-hypertensive treatments.[345] These authors suggested that soluble epoxide hydrolase alters certain oxylipin concentrations, which contributed to the anti-hypertensive effect that was observed in their PAD patient population.[345] In a separate study, Caligiuri *et al.* (2016) evaluated the effects of FLX on central aortic blood pressure in the FlaxPAD population.[344] This study observed a decrease in central systolic and diastolic blood pressures of 10 mmHg and 6 mmHg respectively, in patients with hypertension as compared to placebo.[344] Additionaly, several oxylipins including: 6-keto prostaglandin $F_{1\alpha}$ ($6kPGF_{1\alpha}$), 11,12-dihydroxyeicosatrienoic acid (11,12-DiHETrE), 16-hydroxyeicosatetraenoic acid (16-HETE), 18-HETE, prostaglandin E_2 (PGE_2), and thromboxane

B2 (TXB2), were supported as potential mediators in the anti-hypertensive properties of FLX.[344] Several other clinical studies have cited FLX as possessing potent blood pressure lowering capabilities in male, female, diabetic, dislipidemic, and healthy patient populations.[352-356] These findings were corroberated by Khalesi *et al.* (2015) and Ursoniu *et al.* (2016) who each performed a meta-analysis including a total of 17 different studies that investigated the anti-hypertensive role of FLX.[357, 358]

Continuing with the health benefits associated with FLX, two studies have cited FLX as having potent anti-atherosclerotic effects.[348, 349] Specifically, Bassett *et al.* (2011) fed high fat diets with or without whole ground FLX to low-density lipoprotein receptor-deficient [$LDLr^{(-/-)}$] mice for 14 weeks.[348] The study concluded that FLX was able to protect against trans fatty acid and cholesterol induced atherosclerotic development through FLX's high ALA content.[348] In a separate study, Dupasquier *et al.* (2007) investigated and identified the cellular mechanisms of FLX's anti-atherogenic capabilities in $LDLr^{(-/-)}$ mice.[349] The $LDLr^{(-/-)}$ mice, fed a cholesterol-supplemented diet, exhibited a rise in plasma cholesterol and an increase in atherosclerotic plaque formation.[349] In comparison, supplementation of the cholesterol-enriched diet with 10% ground FLX lowered plasma cholesterol levels, increased plasma ALA content, and inhibited plaque formation in the aorta and aortic sinus.[349] This inhibition of antherosclerosis was characterized by the decrease in expression of the proliferating cell nuclear antigen (PCNA) as well as the inflammatory markers: IL-6, macrophage marker M3/84 (mac-3), and vascular cell adhesion molecule-1 (VCAM-1).[349] A study performed by McCullough *et al.* (2011) cites that the beneficial cardiovascular effects associated with FLX consumption may be due to a change in leptin expression.[350] However, a

meta-analysis performed by Mohammadi-Sartang *et al.* (2017) suggest that these benefits are due to FLX's positive effects on body composition and weight reduction.[350, 351]

In addition to being one of the richest plant sources of ALA, FLX is a source of the potent anti-oxidant lignan: SDG. SDG's anti-oxidative properties stem from its oxygen radical scavenging properties, which function by inhibiting the production of OS.[82, 84, 337-339, 359] The downregulation of OS has led to several cardioprotective effects including: lipid lowering properties, anti-atherosclerotic effects, and anti-hypertensive properties. In a review article by Adolphe *et al.* (2010), it is suggested that a dose of at least 500 mg of SDG per day for 8-weeks is required in order to observe the positive health effects on cardiovascular risk factors in a human population.[360]

A study by Hu *et al.* (2007) evaluated the efficacy of SDG's anti-oxidative properties in different physiological conditions.[337] This study attributed the anti-oxidative properties of SDG to the resonance stabilization of its 3-methoxy-4-hydrowyl component, which occurs in aqueous environments similar to those found *in vivo*.[337] Zhang *et al.* (2008) conducted an 8-week randomized, double-blinded, placebo-controlled study in 55 hypercholesterolaemic subjects to determine the effect of SDG on plasma lipids and fasting glucose levels.[361] This study concluded that the dietary FLX lignan SDG was able to decrease plasma cholesterol and glucose concentrations in a dose-dependent manner.[361] In a separate study conducted by Felmlee *et al.* (2009), a comparative analysis was performed on the effects of the purified FLX lignan SDG and its aglycone metabolite (SECO) in hyperlipidaemic rats.[362] The authors observed a dose-dependent reduction in the rate of body-weight gain, serum total and LDL-cholesterol levels, as well as

hepatic lipid accumulation.[362] Overall, the data suggests that SDG contributes to FLX's hypocholesterolaemic effect.[362]

While SDG has been cited as being a potent lipid lowering and anti-oxidative agent, Prasad (2008) investigated whether or not SDG treatment would regress atherosclerosis.[82] A population of New Zealand white rabbits were assigned to either: i) control (regular diet; n = 6); ii) 0.5% cholesterol diet (2 months; n = 13)); iii) 0.5% cholesterol diet (2 months) then regular diet (2 months; n = 6); iv) 0.5% cholesterol diet (2 months) then regular diet with SDG (2 months; n = 5); or v) 0.5% cholesterol diet (2 months) then regular diet with SDG (4 months; n = 5).[82] This study concluded that: i) regular diet following a high cholesterol diet accelerates atherosclerosis; ii) SDG prevents the progression of atherosclerosis; iii) the prevention of progression of atherosclerosis is associated with a reduction in aortic OS; and iv) a longer duration of treatment with SDG reduces the progression of atherosclerosis to a greater extent.[82]

Overall, FLX has been reported to provide significant health benefits in several cardiovascular diseases. [65, 69, 70, 76, 77, 190, 340-351] However, the scope of FLX's health benefits extends to other diseased conditions including cancer. [85, 363-372] A study by Lowcock *et al.* (2013) investigated the association between the intake of FLX and breast cancer risk.[87] This Canadian study found that FLX intake is associated with a reduction in breast cancer risk and that these findings may be of public health importance with respect to breast cancer prevention.[87] A study by Delman *et al.* (2015) found that SDG may have selective estrogen receptor modulator-like effects that result in anti-estrogenic activity in high estrogen environments.[373] To elaborate, ACI rats (highly susceptibile to estrogen-induced mammary tumors) were administered 0, 10, or 100 ppm of SDG

in their diet while mammary and ovarian cancer progression were induced.[373] This study concluded that treatment with SDG normalized several biomarkers in mammary gland tissue including dysplasia, cell number, and gene expression.[373]

In a randomized, double-blind, placebo-controlled, prospective study, Thompson *et al.* (2005) evaluated the role of FLX on tumor biological markers in postmenopausal patients with primary breast cancer.[363] Patients were randomized to a daily intake of either 25 g of flaxseed-containing muffin (n = 19) or a control (placebo) muffin (n = 13).[363] Two tumor tissue biopsies were performed: i) at baseline and ii) at definitive surgery (30 days) to analyze the rate of tumor cell proliferation, apoptosis, c-erbB2 expression, as well as estrogen and progesterone receptor levels.[363] The results of this study indicated that daily FLX consumption: i) decreased tumor cell proliferation and c-erbB2 expression; ii) increased tumor apoptosis; and iii) increased urinary lignan excretion.[363] Overall, the study concluded that dietary FLX has the potential to reduce tumor growth in patients with breast cancer.[363] In a subset of separate studies performed by Thompson and others, this group not only showed that FLX consumption does not interfere with the cytotoxic abilities of anti-cancer treatments, but that FLX consumption may actually potentiate specific anti-cancer agents like TRZ.[85, 363-372] A recent study conducted by Mason *et al.* (2015) observed that over the course of a 4 week study period, dietary supplementation with FLX oil enhanced the tumor reducing effects of TRZ in an *in vivo* murine model.[85] Despite these encouraging finds on the significant health benefits of FLX in several disease conditions including cancer and cardiovascular disease, little is known about the potential cardioprotective role of FLX in the prevention of DOX+TRZ induced cardiotoxicity.

2.23 Flaxseed in the Prevention of Chemotherapy-Induced Cardiotoxicity

In Canada, both cancer and cardiovascular disease are the leading causes of mortality, accounting for over 120,000 deaths on an annual basis.[1] These two diseases are intricately linked as treatment of cancer may lead to detrimental effects on the heart. Although the current combination of surgical resection, radiation therapy, and chemotherapy may lead to a cure in the early stages of breast cancer, the administration of anti-cancer agents, in particular DOX, is associated with an increased risk of cardiotoxicity.[1] Approximately 25% of breast cancers are positive for the human epidermal growth factor receptor 2 (HER2), which is indicative of a poor prognosis.[1] Although TRZ, a monoclonal antibody against HER2, is routinely used in both the adjuvant and metastatic settings of breast cancer, it is known to potentiate the cardiotoxic side effects of DOX. Women with breast cancer who are receiving DOX+TRZ treatment have an estimated 1 in 4 risk of developing heart failure.[1] As approximately 8000 women in Canada are at risk of developing DOX+TRZ mediated heart failure on an annual basis, there is a major need to prevent this drug induced cardiotoxicity.[1]

Functional foods are defined as having a potentially positive effect on a patient's overall health beyond their basic nutritional components. As functional foods have been reported to provide significant health benefits in several disease conditions including cancer and cardiovascular disease, an increased focus on their cardioprotective role in the evolving field of Cardio-Oncology is needed.[82] In women with breast cancer, complementary and alternative medicine approaches are widely used in an attempt to reduce the overall burden of the disease and prevent recurrence.[83] FLX continues to gain popularity in the health and agri-food industries for these purposes with approximately 30% of breast cancer patients incorporating FLX into their daily use.[83] However, the beneficial effects of FLX in both cancer and cardiovascular disease have been independently

studied. The potential cardioprotective effects of FLX in the combined setting of chemotherapy-induced cardiotoxicity remains to be investigated.

While the study conducted by Mason *et al.* (2015) concluded that FLX oil enhances the cytotoxic abilities of TRZ in an *in vivo* murine model, this group did not specifically study the potential cardioprotective effects of ALA.[85] In a separate study by Yu *et al.* (2013), prophylactic treatment with ALA in DOX treated rats was shown to be partially cardioprotective as it improved LV remodeling, attenuated ROS production, and downregulated cardiomyocyte apoptosis.[374] Although previous studies have demonstrated that ALA has cardioprotective functions in the setting of ischemia, little is known of its effects on chemotherapy induced cardiotoxicity.[374-378]

In addition to ALA, FLX is also the richest dietary source of the potent anti-oxidative plant lignan SDG.[360] Although previous studies have demonstrated that the cardioprotective effects of SDG may be due to its anti-atherogenic, anti-hypertensive, and lipid lowering effects, little is known on its effects in the setting of chemotherapy-induced cardiotoxicity.[82, 360, 361] Yang *et al.* (2016) previously demonstrated that the lignan Schisandrin B (Sch B), which is isolated from the five-flavor berry in China, protects against DOX induced OS injury and cardiotoxicity in both the acute and chronic settings.[83, 359] Of interest, SDG has been shown to possess similar anti-oxidative and oxygen radical scavenging properties as Sch B.[84, 337-339, 359] It is plausible that SDG may be capable of preventing DOX+TRZ cardiotoxicity in the setting of breast cancer. As several studies have already demonstrated that FLX consumption does not possess any adverse side effects, further studies focusing on their potential cardioprotective role in DOX+TRZ mediated cardiotoxicity is warranted.[361, 362, 379]

Chapter 3: Study Rationale, Hypothesis, and Objectives

3.1 Study Rationale

Cancer treatment typically employs a combination of surgery, radiation, and chemotherapy. Recently, an increased understanding of the molecular mechanisms of cancer has led to the development of novel target agents including TRZ, which can be used in combination with DOX for the treatment of breast cancer.[32, 33, 38-42, 44] Despite the effectiveness of these anti-cancer agents, cardiovascular toxicity remains a serious short- and long-term complication of anticancer therapy with DOX+TRZ in the breast cancer setting.[91, 181, 185, 380]

Serial monitoring of LVEF using non-invasive cardiac imaging is the single most important diagnostic tool in the detection of cardiac dysfunction among cancer patients.[56, 248, 381] A reduction in LVEF signifies that irreversible cardiac injury may have occurred.[57, 381] Echocardiographic indices of early LV systolic dysfunction are clinically useful for addressing the cardiac safety profile of anti-cancer drugs, potentially avoiding the detrimental effects of end stage heart failure. Although sensitive echocardiographic techniques including tissue velocity imaging (TVI) and strain imaging may allow for the early detection of LV systolic dysfunction in cancer patients, whether the injury can be prevented at the onset of treatment to improve patient outcomes is of greater concern.[56, 57, 249, 250, 382]

Although FLX is commonly consumed in up to 30% of breast cancer patients to improve overall disease burden and survival, it is important to study whether this natural dietary agent can also reduce the cardiotoxic side effects of DOX+TRZ in the breast cancer setting.[383, 384] The cardioprotective effects of FLX may include its potent anti-inflammatory and anti-oxidative

properties.[84, 337-339] Since this DOX+TRZ mediated cardiotoxicity is multifactorial, including the up-regulation of pro-inflammatory mediators and increased OS production, further research is required to mitigate the adverse side effects of these common anti-cancer drugs.[59, 208]

3.2 Hypothesis

The cardiotoxic side effects of DOX and TRZ in a mouse model will be attenuated by the prophylactic use of FLX by decreasing OS and inflammation thereby conferring a decrease in apoptosis and preservation of overall LV systolic function.

Objective 1: Determine whether the prophylactic administration of FLX and/or its bioactive components ALA and SDG, will attenuate adverse cardiovascular remodeling observed in a chronic *in vivo* murine model of DOX+TRZ mediated cardiotoxicity.

Objective 2: Elucidate the potential mechanism involved in the cardioprotective effects of FLX and/or its components ALA and SDG.

Chapter 4: Materials and Methods

4.1 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/(AC11285).]

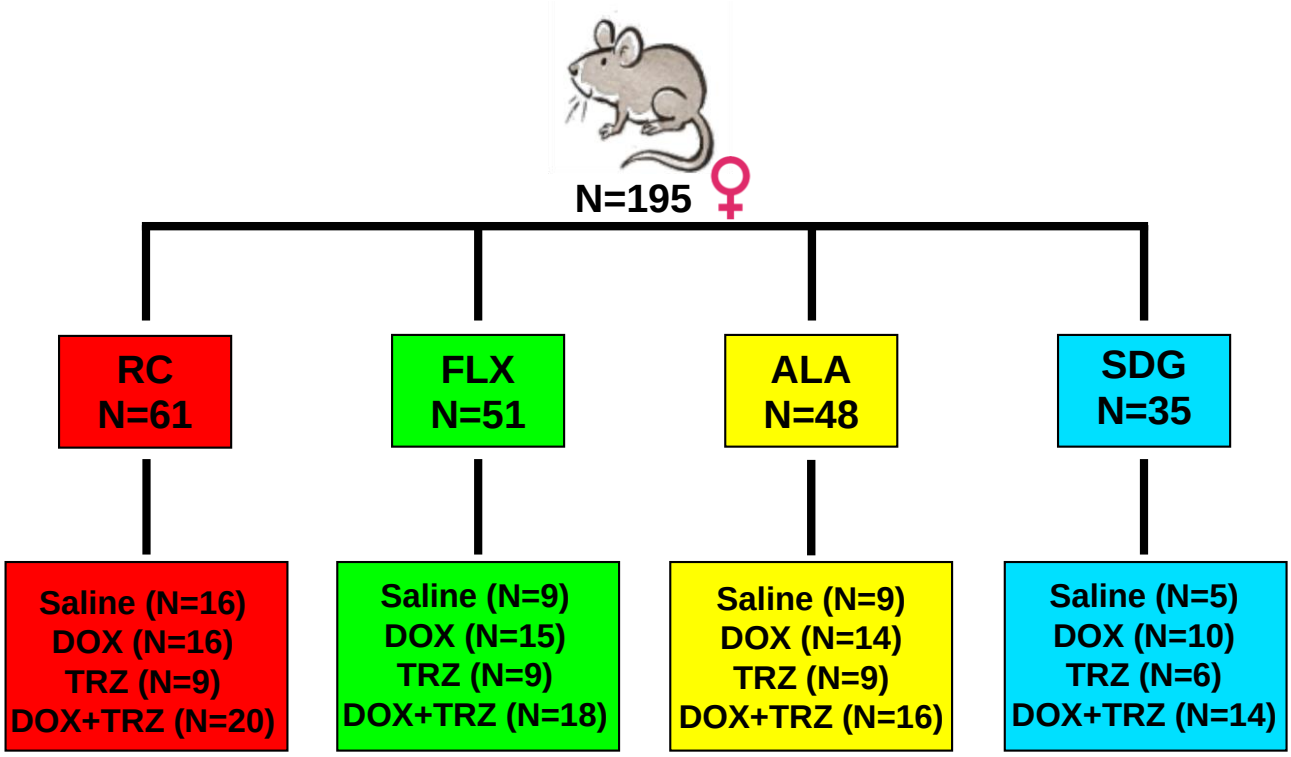
A total of 195 wild-type C57B1/6 female mice (8-12 weeks old; Jackson Laboratories, Bar Harbor, ME, US) were quarantined for 1 week prior to the initiation of the study. All animals were maintained on a 12-hour day/night cycle and received *ad libitum* access to the study diets as well as water during their stay in the animal holding facility. In all animals' baseline TTE, hemodynamics and weight analyses were performed. All mice were randomly assigned to groups, each receiving 1 of 4 daily prophylactic dietary regimens for 6 weeks as indicated in **Figure 10**:

1) Regular Chow (n=61)
2) 10%FLX (n=51)
3) 4.4%ALA (n=48)
4) 0.44%SDG (n=35)

Within each study arm, mice were further randomized to receive weekly intraperitoneal (i.p) injections on weeks 4, 5, and 6 with one of the following agents:

1) 0.9% Saline
2) DOX (8mg/kg)[76]
3) TRZ (3mg/kg)[76]
4) DOX+TRZ (8mg/kg and 3mg/kg, respectively)[76]

Figure 10: Experimental methodology.



A total of 195 WT C57Bl/6 female mice (8-10 weeks old; Jackson Laboratories, ME, US) were randomized into one of four dietary groups to receive the prophylactic administration of RC (n=61); 10%FLX (n=51); 4.4%ALA (n=48); or 0.44%SDG (n=35); on a daily basis for a total of 6 weeks. Mice in each of these dietary groups were further randomized to receive weekly intraperitoneally (i.p) injections of 0.9% saline, DOX (8 mg/kg)[76], TRZ (3 mg/kg)[76], or the combination of DOX+TRZ, on weeks 4, 5, and 6, in order to create a chronic *in vivo* murine model of chemotherapy induced cardiotoxicity.

The cumulative dosages of anti-cancer therapy DOX (24mg/kg) and targeted therapy TRZ (9mg/kg) are the minimum concentrations required to induce a cardiomyopathic state, as previously validated by our group and others.[54, 55, 57, 59-62, 65, 69, 76, 285, 385] The volume of DOX and TRZ were calculated using Equations 2 and 3, respectively.

Equation 2: Volume of Doxorubicin injection.

$$\text{DOX Volume} = \frac{\text{Mouse Weight (kg)} \times \text{Dosage of DOX } (\frac{8\text{mg}}{\text{kg}})}{\text{DOX Concentration } (\frac{2\text{mg}}{\text{mL}})}$$

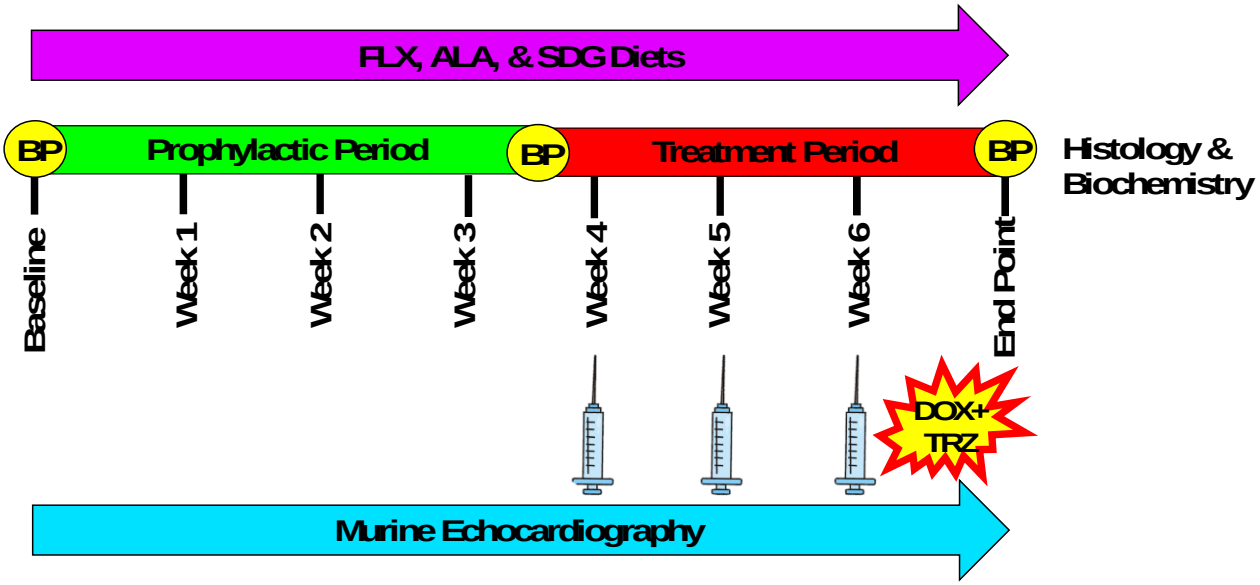
Equation 3: Volume of Trastuzumab injection.

$$\text{TRZ Volume} = \frac{\text{Mouse Weight (kg)} \times \text{Dosage of TRZ } (\frac{3\text{mg}}{\text{kg}})}{\text{TRZ Concentration } (\frac{21\text{mg}}{\text{mL}})}$$

Mice received the RC and various FLX supplementation diets on a daily basis throughout the entire 6-week study period. The FLX supplementation groups were selected due to their anti-inflammatory properties and ability to down-regulate the OS pathways. At the start of weeks 4, 5, and 6, mice received weekly treatment with DOX (8 mg/kg)[76], TRZ (3 mg/kg)[76], or DOX+TRZ intraperitoneally (i.p.) to create a chronic *in vivo* murine model of chemotherapy induced cardiotoxicity.

Serial murine echocardiography and body weight analysis were evaluated on a weekly basis throughout the 6-week study period. Body weights were used to calculate the relative volumes for the DOX (8 mg/kg)[76] and TRZ (3 mg/kg)[76] treatments used in Equation 2 and 3 above. Hemodynamic parameters were evaluated at baseline, the end of week 3 prior to the first i.p. injection of the various treatments, as well as at the end week 6. All animals were then euthanized by an i.p. injection of 150 mg/kg of pentobarbital buffered with 2% lidocaine. Blood samples were collected at this time to be used for the plasma analysis of ALA, SDG metabolites, and oxylipins. The hearts were harvested from the thoracic cavity, rinsed in 0.9% saline, then preserved for further histological and protein analyses. The study timeline is described in **Figure 11**.

Figure 11: Experimental timeline.

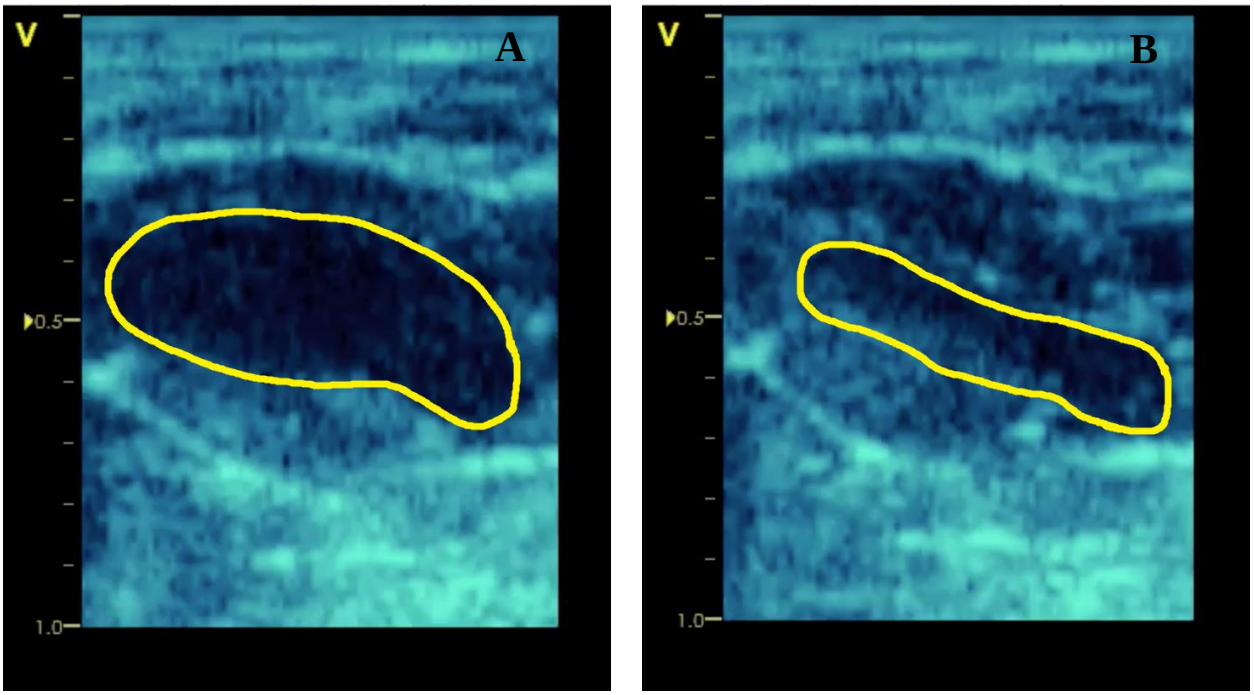


Mice received *ad libitum* access to the RC and various FLX supplementation diets on a daily basis throughout the entire 6-week study period. At the start of weeks 4, 5, and 6, mice received weekly treatment with DOX (8 mg/kg)[76], TRZ (3 mg/kg)[76], or DOX+TRZ intraperitoneally to create a chronic *in vivo* murine model of chemotherapy induced cardiotoxicity.[76] In this *in vivo* model, we studied: i) cardiovascular remodeling using murine echocardiography; ii) hemodynamics; iii) histological injury; iv) inflammatory markers; v) OS; vi) apoptotic markers; vii) plasma content of ALA using gas chromatography and flame ionization detector (GC-FID); viii) plasma content of SDG metabolites using quadrupole time-of-flight mass spectroscopy (Q-TOF-MS); and ix) oxylipins using high performance liquid chromatography and electrospray ionization tandem mass spectrometry (HPLC-ESI-MS). Serial murine echocardiograms were performed on a weekly basis; hemodynamic assessment was performed at baseline, the end of week 3, and end of week 6; plasma was collected at endpoint for GC-FID, Q-TOF, and oxylipin analysis; cardiac tissue was collected at endpoint for histological and biochemical analysis.

4.2 Murine Echocardiography

In vivo assessment of cardiac function was performed using non-invasive murine TTE. All mice were examined at baseline and weekly thereafter for the duration of the 6-week study as described in **Figure 11**. Awake mice underwent echocardiography using a 13-MHz linear array ultrasound probe (Vivid 7, version 11.2, GE Medical Systems, Milwaukee, WI, US). PLAX and short axis (PSAX) windows were evaluated in all mice as previously described.[54, 309] Upon acquisition of PLAX images, endocardial borders of LV cavity were manually traced in order to determine LV end-diastolic and end-systolic volumes used in the calculation of LVEF (Figure 12, Equation 4).

Figure 12: Parasternal long axis view on 2D transthoracic echocardiography.



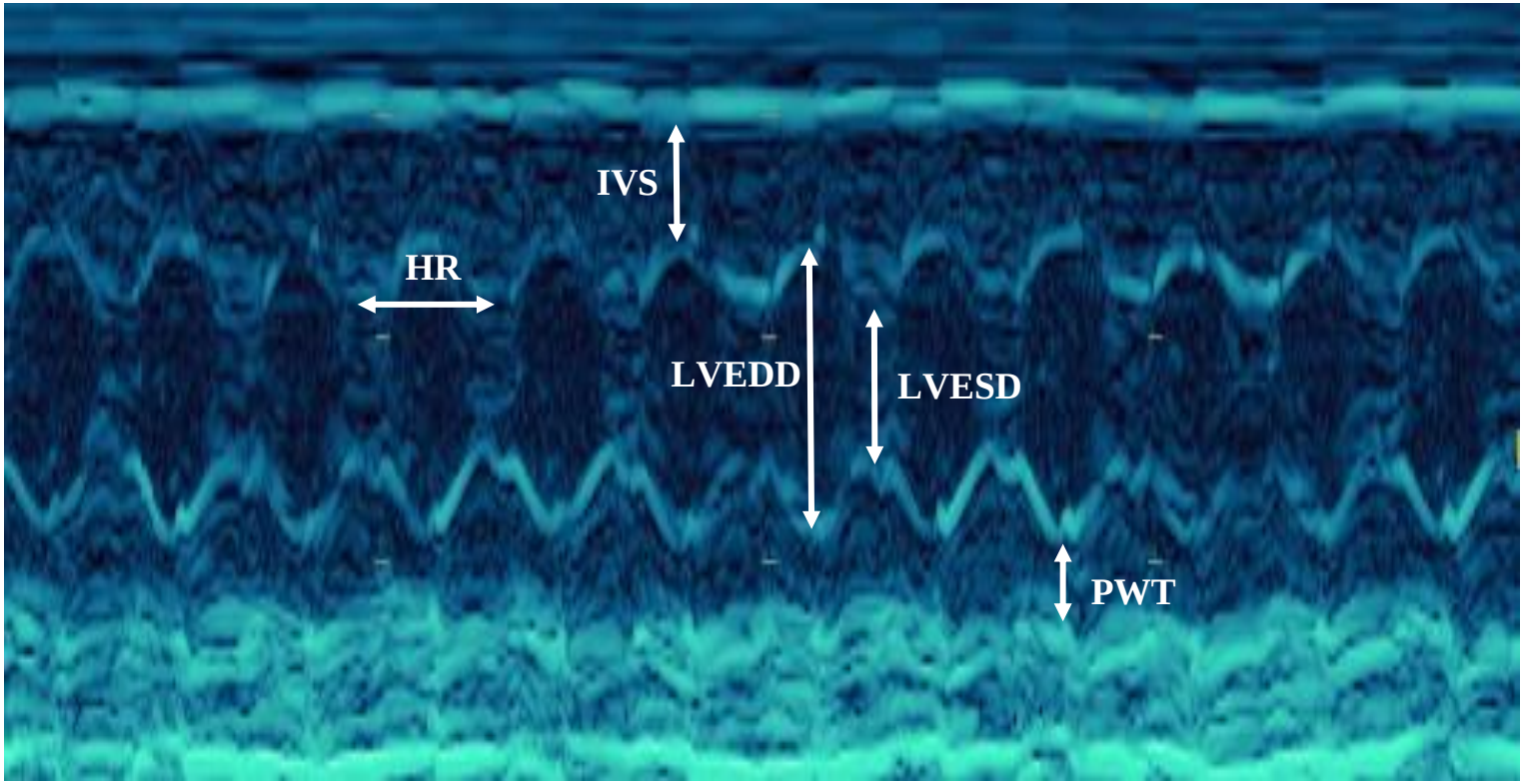
Tracing of the LV on EchoPAC PC software to calculate LVEF. **Panel A:** Tracing of the LV at end diastole. **Panel B:** Tracing of the LV at end systole. LV, Left ventricle; LVEF, Left ventricular ejection fraction.

Equation 4: Left Ventricular Ejection Fraction.

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

PSAX windows were recorded to derive the motion mode (M-mode) echocardiographic parameters (Figure 13), including LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), posterior wall thickness (PWT), and interventricular septal thickness (IVS). Tissue Doppler Imaging (TDI) was acquired on a PSAX view at the level of the papillary muscles, at a rate of 483 frames per second. Peak systolic endocardial velocity (V_{ENDO}) was obtained from a region of interest of 0.2 mm x 0.2 mm on the posterior wall.[57, 381] 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. Echocardiographic data collection and analysis were conducted by observers blinded to the various treatment groups.

Figure 13: M-mode view on 2D transthoracic echocardiography.



Measuring echocardiographic parameters using M-mode on EchoPAC PC software. HR, heart rate; IVS, interventricular septum; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; M-mode, motion mode; PWT, posterior wall thickness.

To assess the variability of LV cavity dimensions and function, a total of 30 mice were randomly chosen from the various treatment groups. A single observer (DSJ) performed independent measurements of LVEDD and LVEF on two separate days two weeks apart in order to evaluate intra-observer variability. Inter-observer variability was determined separately by two independent observers (CYA and DSJ). Intra- and inter- observer variations were defined as the difference between the two observations divided by the mean of the observations and expressed as absolute numbers.

4.3 Histological Analysis and Cardiac Fibrosis

In the preparation for electron microscopy, 3% glutaraldehyde in 0.1M phosphate buffer at pH 7.3 was used to fix the heart tissues for 3 hours at room temperature. They 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.[386] 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.

The presence of fibrosis was analyzed by collecting the hearts from control mice and the various treatment groups. Specimens were fixed in 10% buffered formalin for 24 hours and then processed

for paraffin embedding. Serial 5 μm thick sections were cut from each heart for dewaxing and rehydration and then were stained with Masson's Trichrome solution to detect fibrosis. With this stain, the normal myofiber was stained red, while the collagen was stained blue. Digital pictures were taken with identical exposure settings for all the sections.

4.4 Hemodynamics

Non-invasive HR and blood pressure (BP) measurements including systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) were recorded in non-sedated, restrained mice using a tail cuff method (CODA system, High Throughput, Kent Scientific, Torrington, CT), as previously reported and successfully completed in our laboratory.[62, 64, 309, 387] Briefly, the holding platform was heated to 30°C, and 15 consecutive blood pressure readings were recorded with 1-minute rest intervals between the readings. The mean scores of 15 true blood pressure readings were included in the data set. Blood pressure was measured at baseline, at the end of week 3 prior to the first i.p. injection of the various treatment groups, as well as at the end of week 6 before the mice were sacrificed. Pulse pressures were calculated from the diastolic and systolic pressure values using Equation 5. Pulse pressures were then used in Equation 6 to calculate MAP of each mouse. The average values for SBP were computed using 9 individual readings.

Equation 5: Pulse Pressure.

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

Equation 6: Mean Arterial Pressure.

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

4.5 Gas Chromatography and Flame Ionization Detection

Blood samples were collected once mice were sacrificed and aliquoted into Microvette CB 300 K2E tubes (Product #: 16.444.100, Sarstedt AG & Co.), inverted, and then placed on ice for 30 minutes. Samples were centrifuged at 4500G for 5 mins at room temperature and the plasma was collected. Using a Socorex with a glass pipette, 50 µL of plasma, 50 µL H_2O, and 100 µL of internal standard were added into a 10 mL screw top culture tube (Product #: 9450660009, Kimble). 1 mL of toluene followed by 1.2 mL of methanolic hydrochloric acid (HCl) was added into Tube A and vortexed for 30 seconds. The tubes were incubated in a Fisher Isotemp oven (100 Series Model 116G) at 80°C for 90 minutes. After the samples were allowed to cool for 15 minutes, 1 mL of deionized water (ddH_2O) was added into the tubes and vortexed for 15 seconds. Samples were then centrifuged (Eppendorf Centrifuge 5804R 15amp Version) at 4500G at 4°C for 15 minutes with the break on low. The top layer was transferred into a second tube labelled B. Tube A samples were re-spun a second time at 4500G at 4°C for 15 minutes with the break on low to collect the sample that may have been reabsorbed by the gel layer. 1 mL of petroleum ether was added to the bottom layer of Tube A, vortexed for 15 seconds then centrifuged at 4500G at 4°C for 15 minutes with the break on low. The top layer of Tube A was transferred to Tube B. Again, Tube A samples were re-spun at 4500G at 4°C for 15 minutes with the break on low to collect the sample that was reabsorbed by the gel layer. 2 mL of ddH_2O was added to the combined layers in

Tube B, vortexed for 15 seconds then centrifuged at 4500G at 4°C for 15 minutes with the break on low. The top layer of Tube B was transferred to Tube C and dried under Nitrogen using the dry glass bead unit with the heat on. Once the solvent has evaporated, using a Drummond Microdispenser (The Drummond Scientific Co., Broomall, PA, USA) and 100 μL – 200 μL Microdispenser tubes (Product #: 3-000-275-G, The Drummond Scientific Co.), 200 μL of hexane was immediately added into Tube C and the samples were re-suspended. Samples were aliquoted into a gas chromatography (GC) vial (Product #: V0808-830, Canadian Life Science) with a conical insert bottom spring (Product #: I025BS-629, Canadian Life Science). The GC vials were sealed with a 9mm red screw cap (Product #: C395E-09SR, Canadian Life Science) and stored in a -20°C freezer overnight.

The mHCl-methylated samples were separated on a DB225MS column with dimensions of 30 m X 0.25 mm diameter and 0.25 μm film thickness (Agilent Technologies Canada Inc., Mississauga, Ontario) using a Varian 450-GC with flame ionization detection (FID) (Agilent Technologies Canada Inc., Mississauga, Ontario), as previously described.[388, 389] The temperature was set according to the follow program with a total run time of 46.67 minutes; i) the temperature was set at 70°C for 2 minutes, then raised to 180°C at 30°C/min and held for 1 minute; ii) the temperature was then raised to 200°C at 10°C/min, and held for 2 minutes; iii) the temperature was raised to 220°C at 2°C/min and held for 10 minutes; iv) finally the temperature was raised to 240°C at 20°C/min and held for 15 minutes. The samples were run with a 20:1 split ratio and a 1.3ml/min column flow. Hydrogen was used as the carrier gas.

4.6 Quadrupole Time-of-Flight and Mass Spectroscopy

Blood samples were collected once mice were sacrificed and aliquoted into Microvette CB 300 K2E tubes (Product #: 16.444.100, Sarstedt AG & Co.), inverted, and then placed on ice for 30 minutes. Samples were centrifuged at 4500G for 5 mins at room temperature and the plasma was collected. In preparation for the metabolomics analysis, 500 µL of plasma was aliquoted into 2 mL Eppendorf tubes. Next, 1 mL of methanol (MeOH) was added to the plasma and vortexed for 5 minutes at 1500 RPM. The samples were then centrifuged for 30 seconds at 10000G. The samples were then filtered through a 0.2 µm Millex-GN Nylon membrane for fine particles and dried under Nitrogen flow. Once dried, the samples were then reconstituted in 200 µL of a 3:1 ratio of MeOH:H_2O. The samples were run on a HPLC system (1290 Infinity Agilent Ltd., Santa Clara, CA, USA) including a binary pump, degasser, well-plate auto-sampler, and column [Extend-C18, 2.1 x 50 mm, 1.8 um, 600 bar (Agilent Technologies, Santa Clara, CA, USA)]. A 6538 UHD Accurate LC-QTOF-MS (Agilent Technologies, Santa Clara, CA, USA) with dual electro-spray ionization (ESI) source was used to detect the metabolites, as previously described.[390-392] The MS parameters were set to the following: capillary voltage (4000 V); fragmentor (175 V); drying gas: N_2 utilized at 11 L/min at 300˚C; nebulizer settings: 50 psi; MS spectra range: 50-1700 m/z; and known reference masses: 121.0508 and 922.0097 for (ESI+), 112.9860 and 1033.9880 for (ESI-). The mobile phases used were: A) Water + 0.1% formic acid; and B) acetonitrile + 0.1% formic acid. The column temperature was set to 45˚C and the run time was set to 13 minutes. The gradients were set as followed: i) 0.5 mins 70% A; 30% B; ii) 12.5 mins 0% A; 100% B; and iii) 13 mins 70% A; 30% B. Post-run time was 1 min prior to the injection of the next sample, and the flow rate was set at 0.4 mL/min. MS data acquisitions were in both the positive (+) and negative (-) electrospray ionization (ESI) modes.

4.7 Oxylipin Analysis

In preparation for the oxylipin analyses, a total of 1 mL of water pH 3.0, 100 μL of internal standard, and 100 μL of plasma were aliquoted into a 2 mL Eppendorf tube. Samples were quickly vortexed, and then the pH tested using indicator strips. If necessary, the samples were acidified to pH 3.0 with approximately 5 μL of 1N HCl. If the pH dropped below 3.0, 1N sodium hydroxide (NaOH) was used to raise the pH back to 3.0. Samples were then centrifuged at 14000 RCF at 4°C for 10 minutes to remove debris. Strata-X SPE (Phenomenex, 33μ, 60 mg/3 mL) columns for each sample were arranged on a wooden rack and used to extract the oxylipins. Columns were first conditioned by aliquoting 3.5 mL of MeOH through each column. The columns were equilibrated by pushing 3.5 mL of pH 3.0 water through each column using a BD 10 mL syringe. Samples were then loaded into the columns. 1 mL of 10% MeOH in pH 3.0 water was added to the sample vials, vortexed, then centrifuged at 14000 RCF at 4°C for 5 minutes to collect any remaining sample. These samples were then added to the columns and pushed through as previously described. The columns were washed with 2 mL of pH 3.0 water. The columns were dried by pushing through 1 mL of Hexane. Once dried, 1.5 mL microtubes were placed underneath each column containing the sample. The columns were then eluted using 1 mL of MeOH. Specifically, pressure was applied to allow the MeOH to enter the sorbent where it was left to soak for 1 minute. The MeOH was then pushed through to collect the samples. Excess air was displaced using Nitrogen gas (N_2) before the samples were vortexed, spun down, and stored at -20°C.

All samples were dried under N_2. Specifically, Eppendorf tubes containing the samples were thawed and then placed into the evaporator (37°C). Needles were lowered to allow N_2 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 N_2, 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).[393] 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.[393] 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 the delustering potentials (-30 to -100 V) varied according to molecular species.[393]

4.8 Western Blotting

Frozen heart tissues of the mice were ground in the presence of liquid nitrogen into powder. Tissues were homogenized in the 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 #: 88667) inhibitors (Thermo Scientific) were added to the RIPA buffer prior to its use. 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. The total concentration of protein 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 (SDS-PAGE) was used to separate 30 μg of protein at 55 mA for 90 minutes using the large gel system. The proteins were transferred at 100 V for 60 minutes at 10°C on to a 0.2 μm pore size polyvinylidene fluoride (PVDF) membrane (Product #: 88520, Thermo Scientific). The membranes were blocked for 60 minutes at room temperature using 5% skim milk powder or BSA (Product #: 0332-100G, Thermo Scientific) in 1X Tris Buffered Saline with 0.1% Tween 20 (TBST). The membranes were probed overnight at 4°C with primary antibodies specific to PARP, Bax, Bcl-X_L, Caspase-3, TNF-α, NF-κβ, IL-1β, IL-6, Bnip3 and GAPDH (Product #: 9542; 2772; 2762; 9662; 3707; 8242; 12242; 12912; 3769 and 2118; Cell Signaling Technology). The primary antibody specific to PPAR-α was ordered from Abcam (ab24509). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (BioRad) was added to the membrane and left to probe for 60 minutes. A Pierce ECL Western Blotting Substrate (Product #: 32106, Thermo Scientific) was used to detect protein bands on CL-Xposure blue X-ray film (Product #: XC6A2, Mandel Scientific Company Inc.). Densitometric analysis using QuantityOne software (BioRad) was used to quantify band intensities normalized to GAPDH, which was used as the loading control.

4.9 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 5: Results

Murine Echocardiography: DOX Treatment

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

In the animals treated with RC+DOX, LVEDD significantly increased from 2.8±0.2 mm at baseline to 3.8±0.1 mm by week 6 of the study (Figure 14). However, the prophylactic administration of FLX, ALA, or SDG partially attenuated this increase in LVEDD, with values of 3.3±0.1 mm, 3.2±0.1 mm, and 3.3±0.1 mm, respectively at week 6 ($p<0.05$).

Similarly, treatment with RC+DOX led to the development of LV systolic dysfunction as LVEF significantly decreased from 75±2% at baseline to 49±2% at week 6 of the study (Figure 15). However, the prophylactic administration of FLX, ALA or SDG was partially cardioprotective with LVEF values of 66±3%, 63±3% and 65±4%, respectively at week 6 ($p<0.05$).

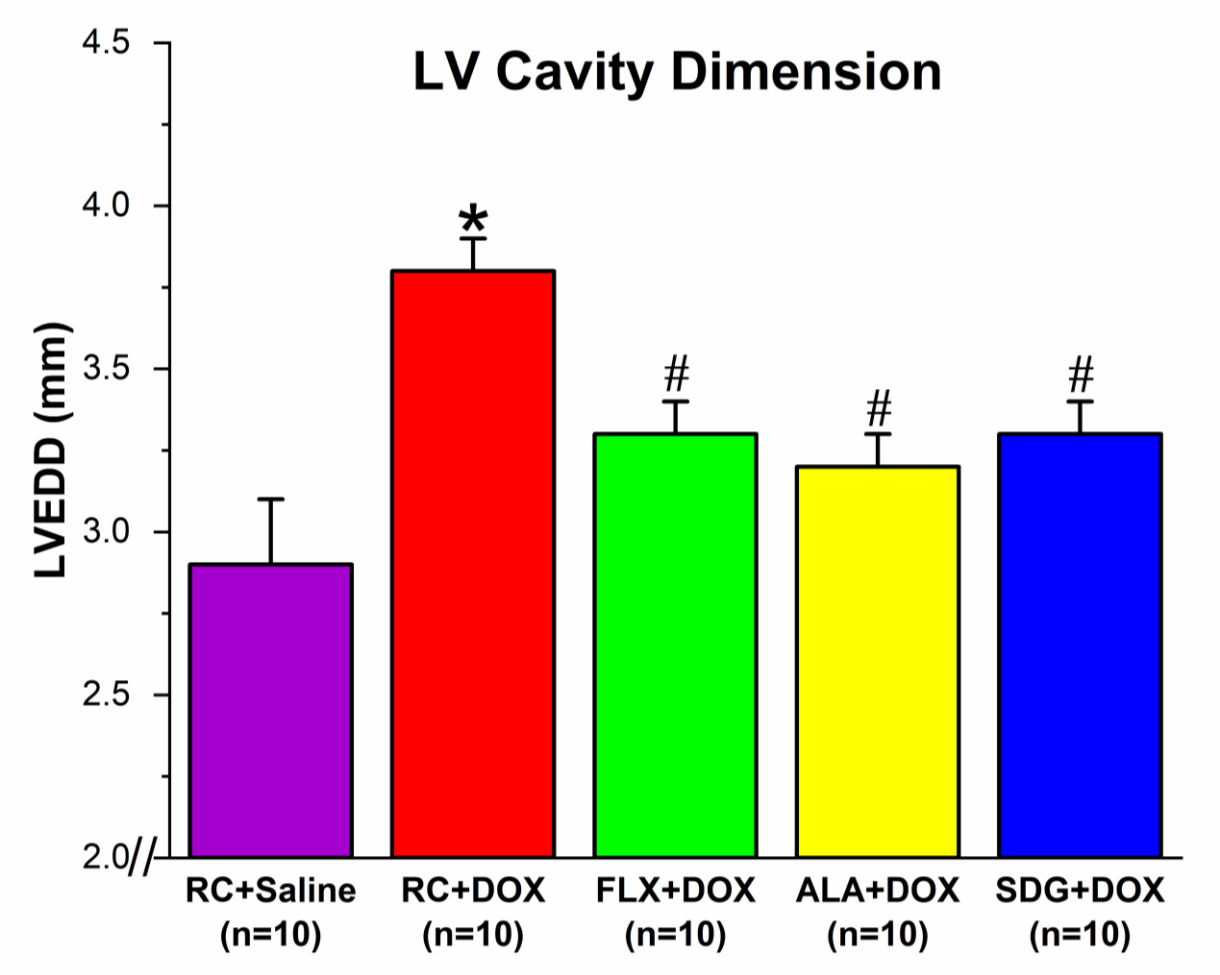
Table 2: Echocardiographic data from C57Bl/6 female mice treated with Saline or DOX with or without prophylactic FLX, ALA, or SDG diets from baseline to week 6.

Variable	Group	Baseline	Week 6	p value
HR (beats/min)	RC+Saline (n=10)	688±7	697±9	0.82
	RC+DOX (n=10)	679±9	688±9	0.77
	FLX+DOX (n=10)	702±8	698±8	0.83
	ALA+DOX (n=10)	685±8	694±6	0.71
	SDG+DOX (n=10)	691±9	683±11	0.81
IVS (mm)	RC+Saline (n=10)	0.81±0.01	0.82±0.02	0.89
	RC+DOX (n=10)	0.80±0.01	0.82±0.02	0.89
	FLX+DOX (n=10)	0.82±0.01	0.81±0.03	0.93
	ALA+DOX (n=10)	0.82±0.01	0.81±0.02	0.92
	SDG+DOX (n=10)	0.82±0.01	0.81±0.02	0.91
PWT (mm)	RC+Saline (n=10)	0.81±0.03	0.81±0.02	0.93
	RC+DOX (n=10)	0.82±0.02	0.83±0.02	0.91
	FLX+DOX (n=10)	0.82±0.01	0.81±0.02	0.93
	ALA+DOX (n=10)	0.82±0.01	0.81±0.02	0.89
	SDG+DOX (n=10)	0.83±0.01	0.82±0.02	0.92
LVEDD (mm)	RC+Saline (n=10)	2.8±0.2	2.9±0.2	0.84
	RC+DOX (n=10)	2.8±0.2	3.8±0.1*	<0.05
	FLX+DOX (n=10)	2.8±0.2	3.3±0.1$^{\#}$	<0.05
	ALA+DOX (n=10)	2.9±0.1	3.2±0.1$^{\#}$	<0.05
	SDG+DOX (n=10)	2.9±0.2	3.3±0.1$^{\#}$	<0.05
LVEF (%)	RC+Saline (n=10)	73±2	72±4	0.86
	RC+DOX (n=10)	75±2	49±2*	<0.05
	FLX+DOX (n=10)	74±2	66±3$^{\#}$	<0.05
	ALA+DOX (n=10)	73±3	63±3$^{\#}$	<0.05
	SDG+DOX (n=10)	72±2	65±4$^{\#}$	<0.05

ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; Heart Rate (HR); Interventricular Septal Thickness (IVS); Left Ventricular End-Diastolic Diameter (LVEDD); Left Ventricular Ejection Fraction (LVEF); Posterior Wall Thickness (PWT); RC, Regular Chow; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside.

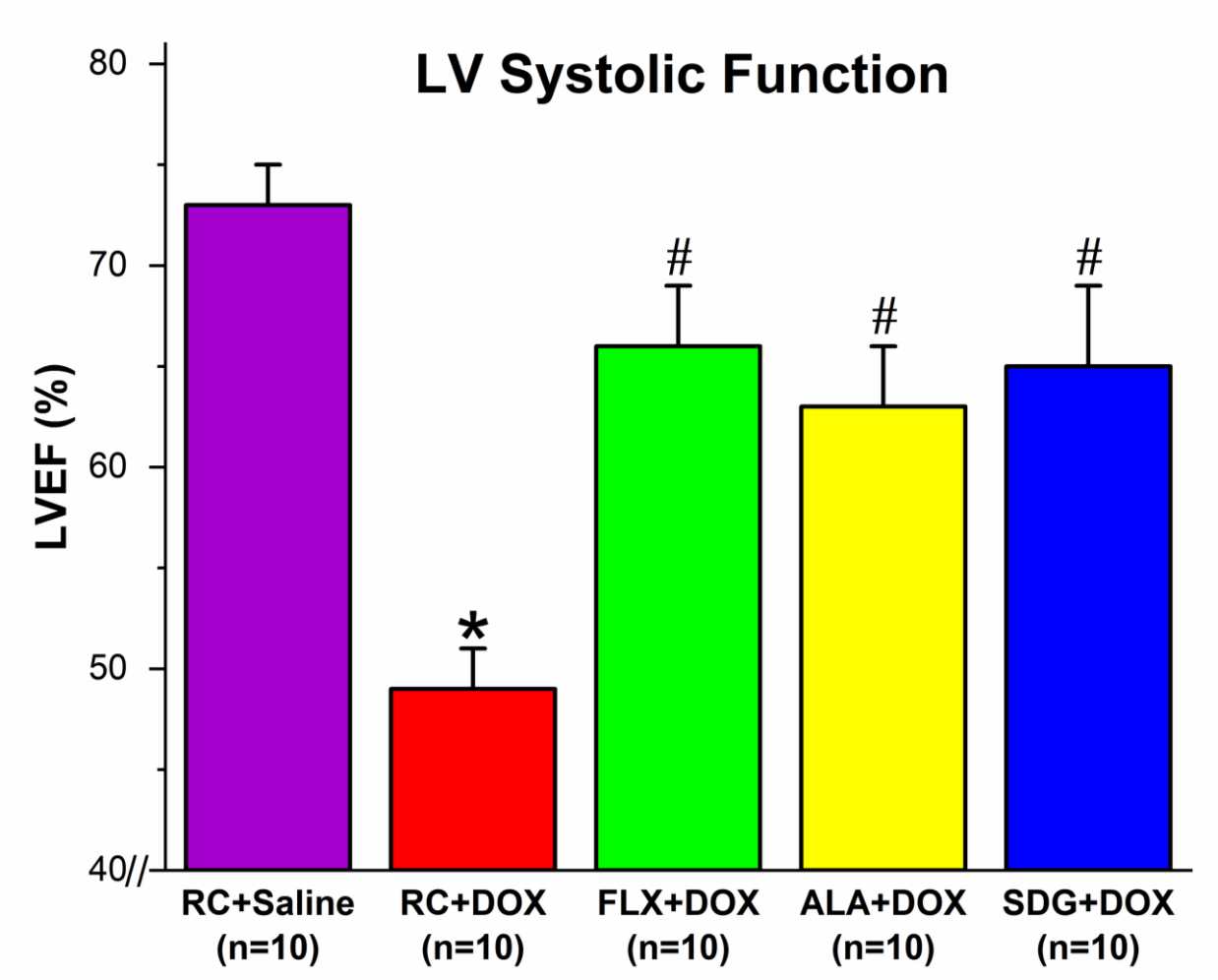
The values are presented as mean ± SD. *$p<0.05$ RC+Saline vs. RC+DOX. #$p<0.05$ RC+DOX vs. FLX+DOX, ALA+DOX, or SDG+DOX.

Figure 14: Changes in LVEDD in DOX treated mice receiving prophylactic treatment with FLX, ALA, or SDG.



C57Bl/6 female mice treated with RC+DOX developed a significant increase in LVEDD values at week 6. LVEDD values significantly improved with the prophylactic administration of FLX, ALA, and SDG in animals receiving DOX. The results are reported as mean ± SD. *$p<0.05$ RC+Saline vs. RC+DOX. #$p<0.05$ RC+DOX vs. FLX+DOX, ALA+DOX, or SDG+DOX. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; LV, Left Ventricle; LVEF, Left Ventricular End Diastolic Diameter; RC, Regular Chow; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside.

Figure 15: Changes in LVEF in DOX treated mice receiving prophylactic treatment with FLX, ALA, or SDG.



C57Bl/6 female mice treated with RC+DOX developed a significant decrease in LVEF values at week 6. LVEF values significantly improved with the prophylactic administration of FLX, ALA, and SDG in animals receiving DOX. The results are reported as mean ± SD. *$p<0.05$ RC+Saline vs. RC+DOX. #$p<0.05$ RC+DOX vs. FLX+DOX, ALA+DOX, or SDG+DOX. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; LV, Left Ventricle; LVEF, Left Ventricular Ejection Fraction; RC, Regular Chow; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside.

Murine Echocardiography: DOX+TRZ Treatment

At baseline, all echocardiographic parameters including HR, IVS, PWT, LVEDD, and LVEF, were similar between all treatment groups (Table 3). HR, IVS, and PWT remained within the normal range for all the treatment groups throughout the duration of the 6-week study.

In the animals treated with RC+DOX+TRZ, LVEDD significantly increase from 2.8±0.3 mm at baseline to 4.1±0.2 mm by week 6 of the study (Figure 16). However, the prophylactic administration of FLX, ALA, or SDG partially attenuated this increase in LVEDD, with values of 3.4±0.1 mm, 3.3±0.1 mm, and 3.4±0.2 mm, respectively at week 6 ($p<0.05$).

Similarly, treatment with the combination of RC+DOX+TRZ led to the development of cardiotoxicity as LVEF values significantly decreased from 74±3% at baseline to 38±2% at week 6 of the study (Figure 17). However, the prophylactic administration of FLX, ALA or SDG was partially cardioprotective with LVEF values of 61±2%, 60±3% and 61±4%, respectively at week 6 ($p<0.05$). All echocardiographic parameters remained within the normal range in the mice who were treated with TRZ throughout the entire duration of the 6-week study (data not shown).

As shown in Table 4, there was minimal intra-observer and inter-observer variability for the LVEDD and LVEF measurements of DOX only and DOX+TRZ treated mice, as summarized in Table 4.

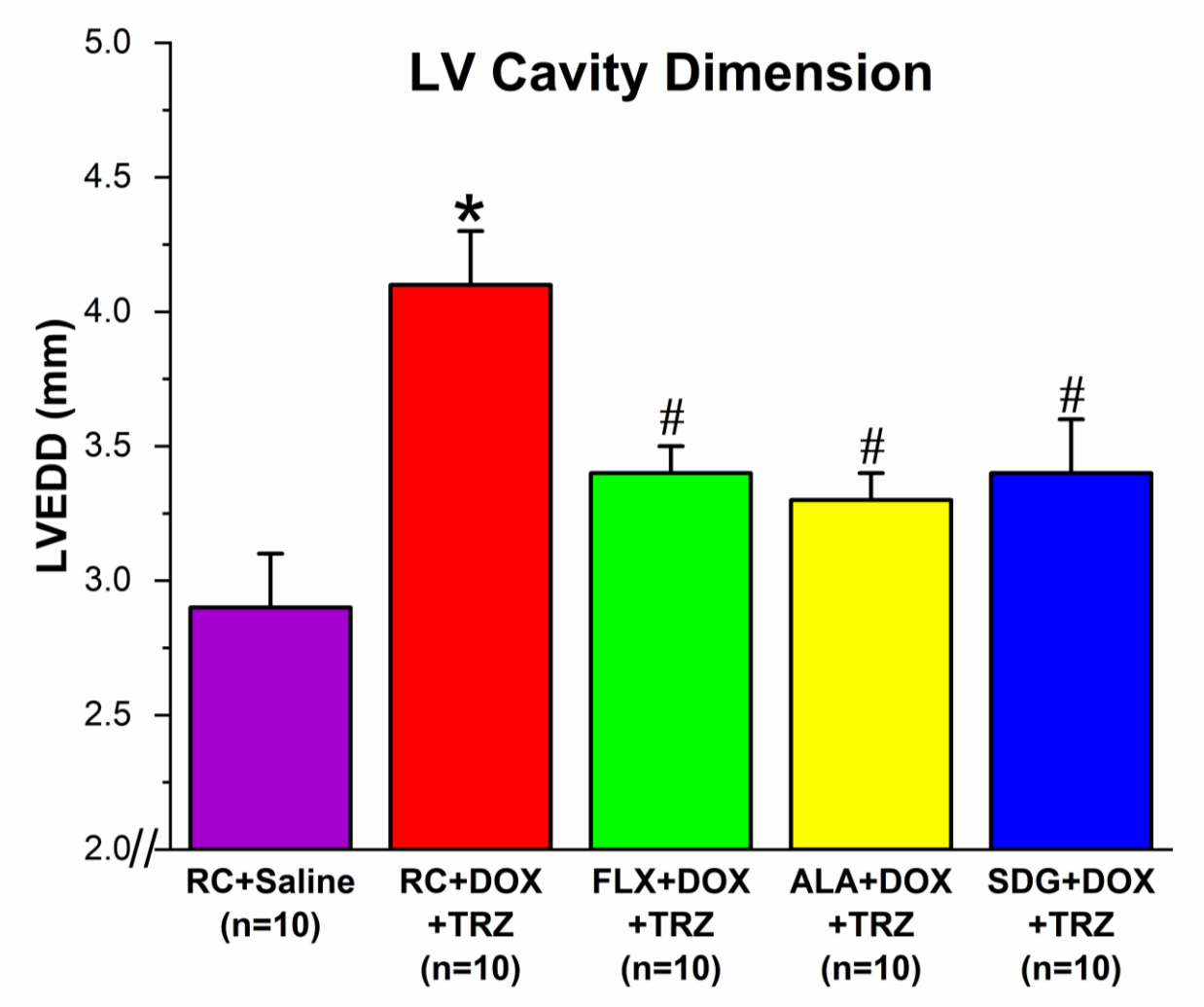
Table 3: Echocardiographic data from C57Bl/6 female mice treated with Saline or DOX+TRZ with or without prophylactic FLX, ALA, or SDG diets from baseline to week 6.

Variable	Group	Baseline	Week 6	p value
HR (beats/min)	RC+Saline (n=10)	688±7	697±9	0.82
	RC+DOX+TRZ (n=10)	684±7	694±11	0.72
	FLX+DOX+TRZ (n=10)	698±7	702±6	0.88
	ALA+DOX+TRZ (n=10)	691±6	698±7	0.83
	SDG+DOX+TRZ (n=10)	685±6	687±11	0.88
IVS (mm)	RC+Saline (n=10)	0.81±0.01	0.82±0.02	0.89
	RC+DOX+TRZ (n=10)	0.81±0.01	0.82±0.02	0.88
	FLX+DOX+TRZ (n=10)	0.82±0.01	0.82±0.02	0.95
	ALA+DOX+TRZ (n=10)	0.81±0.01	0.81±0.02	0.94
	SDG+DOX+TRZ (n=10)	0.82±0.01	0.83±0.01	0.91
PWT (mm)	RC+Saline (n=10)	0.81±0.03	0.81±0.02	0.93
	RC+DOX+TRZ (n=10)	0.81±0.02	0.82±0.02	0.92
	FLX+DOX+TRZ (n=10)	0.83±0.01	0.82±0.02	0.91
	ALA+DOX+TRZ (n=10)	0.81±0.02	0.82±0.02	0.88
	SDG+DOX+TRZ (n=10)	0.82±0.02	0.82±0.04	0.95
LVEDD (mm)	RC+Saline (n=10)	2.8±0.2	2.9±0.2	0.84
	RC+DOX+TRZ (n=10)	2.8±0.3	4.1±0.2*	<0.05
	FLX+DOX+TRZ (n=10)	2.8±0.1	3.4±0.1$^{\#}$	<0.05
	ALA+DOX+TRZ (n=10)	2.8±0.2	3.3±0.1$^{\#}$	<0.05
	SDG+DOX+TRZ (n=10)	2.8±0.2	3.4±0.2$^{\#}$	<0.05
LVEF (%)	RC+Saline (n=10)	73±2	72±4	0.86
	RC+DOX+TRZ (n=10)	74±3	38±2*	<0.05
	FLX+DOX+TRZ (n=10)	74±2	61±2$^{\#}$	<0.05
	ALA+DOX+TRZ (n=10)	73±3	60±3$^{\#}$	<0.05
	SDG+DOX+TRZ (n=10)	72±2	61±4$^{\#}$	<0.05

ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; Heart Rate (HR); Interventricular Septal Thickness (IVS); Left Ventricular End-Diastolic Diameter (LVEDD); Left Ventricular Ejection Fraction (LVEF); Posterior Wall Thickness (PWT); RC, Regular Chow; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside; TRZ, Trastuzumab.

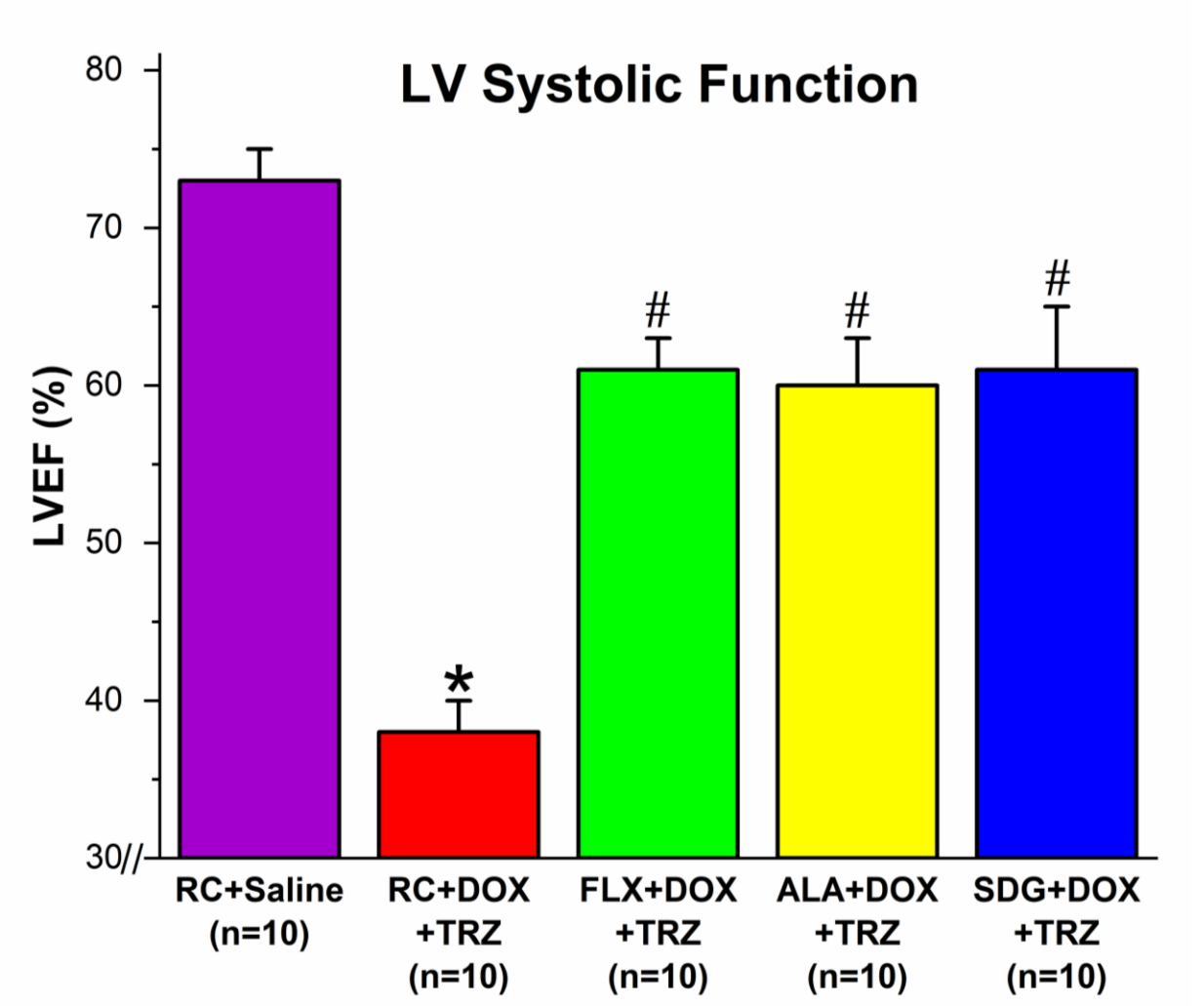
The values are presented as mean ± SD. *$p<0.05$ RC+Saline vs. RC+DOX+TRZ. #$p<0.05$ RC+DOX+TRZ vs. FLX+DOX+TRZ, ALA+DOX+TRZ, or SDG+DOX+TRZ.

Figure 16: Changes in LVEDD in DOX+TRZ treated mice receiving prophylactic treatment with FLX, ALA, or SDG.



C57Bl/6 female mice treated with RC+DOX+TRZ developed a significant increase in LVEDD values at week 6. LVEDD values significantly improved with the prophylactic administration of FLX, ALA, and SDG in animals receiving DOX. The results are reported as mean ± SD. *$p<0.05$ RC+Saline vs. RC+DOX+TRZ. #$p<0.05$ RC+DOX+TRZ vs. FLX+DOX+TRZ, ALA+DOX+TRZ, or SDG+DOX+TRZ. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; LV, Left Ventricle; LVEF, Left Ventricular End Diastolic Diameter; RC, Regular Chow; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside; TRZ, Trastuzumab.

Figure 17: Changes in LVEF in DOX+TRZ treated mice receiving prophylactic treatment with FLX, ALA, or SDG.



C57Bl/6 female mice treated with RC+DOX+TRZ developed a significant decrease in LVEF values at week 6. LVEF values significantly improved with the prophylactic administration of FLX, ALA, and SDG in animals receiving DOX. The results are reported as mean ± SD. *$p<0.05$ RC+Saline vs. RC+DOX+TRZ. #$p<0.05$ RC+DOX+TRZ vs. FLX+DOX+TRZ, ALA+DOX+TRZ, or SDG+DOX+TRZ. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; LV, Left Ventricle; LVEF, Left Ventricular Ejection Fraction; RC, Regular Chow; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside; TRZ, Trastuzumab.

Table 4: Intra- and inter-observer variabilities of the echocardiographic parameters.

Echocardiographic parameters	**Mean difference ± SD**
Intra-observer variability	
LVEDD, mm	0.04±0.01
LVEF, %	0.6±0.2
Inter-observer variability	
LVEDD, mm	0.05±0.01
LVEF, %	1.3±0.2

Assessments of intra- and inter-observer variabilities in C57Bl/6 female mice treated with Saline or DOX+TRZ with or without prophylactic FLX, ALA, or SDG diets from baseline to week 6. The values are presented as mean ± SD. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; RC, Regular Chow; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside; TRZ, Trastuzumab.

Histological Analysis and Cardiac Fibrosis

Approximately 15,000 cells were scanned from 3 random blocks of tissue and assessed for cellular integrity. Normal cell morphology was observed in RC+Saline treated mice (Figure 18A). However, in mice treated with RC+DOX, there was significant disruption of the myofibrils, vacuolization, and loss of sarcomere integrity of the cardiomyocytes (Figure 18B). The prophylactic administration of FLX or ALA significantly attenuated the disruption of the myofibrils caused by DOX ($p<0.006$ and $p<0.009$, respectively) (Figure 18C-D). However, prophylactic treatment with SDG did not significantly prevent cellular damage caused by DOX ($p<0.40$).

In comparison to mice receiving DOX alone, no significant changes in cellular integrity were observed in the mice receiving TRZ alone at week 6. Prophylactic administration of FLX, ALA, or SDG did not significantly alter cellular integrity as compared to RC+Saline treated mice at the end of the study (data not shown). However, treatment with DOX+TRZ demonstrated increased cardiotoxicity with more pronounced myofibril damage (Figure 19B). The prophylactic administration of FLX, ALA, and SDG was partially cardioprotective at preserving myofibril integrity at week 6 in mice receiving DOX+TRZ ($p<0.019$, $p<0.033$, and $p<0.002$, respectively) (Figure 19C-E). Masson's trichrome staining did not detect fibrosis in any of the treatment groups (data not shown).

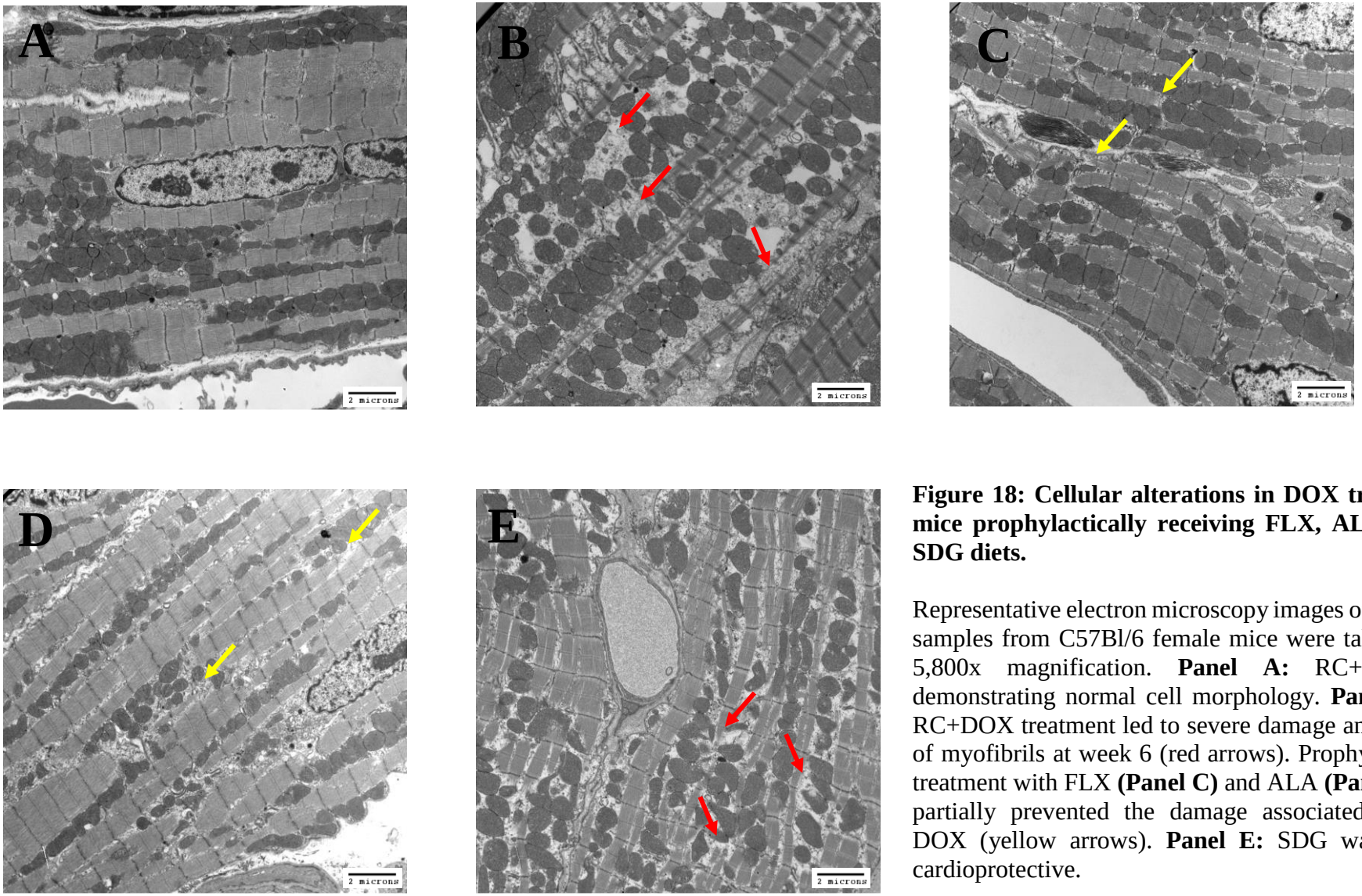


Figure 18: Cellular alterations in DOX treated mice prophylactically receiving FLX, ALA, or SDG diets.

Representative electron microscopy images of heart samples from C57Bl/6 female mice were taken at 5,800x magnification. **Panel A:** RC+Saline demonstrating normal cell morphology. **Panel B:** RC+DOX treatment led to severe damage and loss of myofibrils at week 6 (red arrows). Prophylactic treatment with FLX **(Panel C)** and ALA **(Panel D)** partially prevented the damage associated with DOX (yellow arrows). **Panel E:** SDG was not cardioprotective.

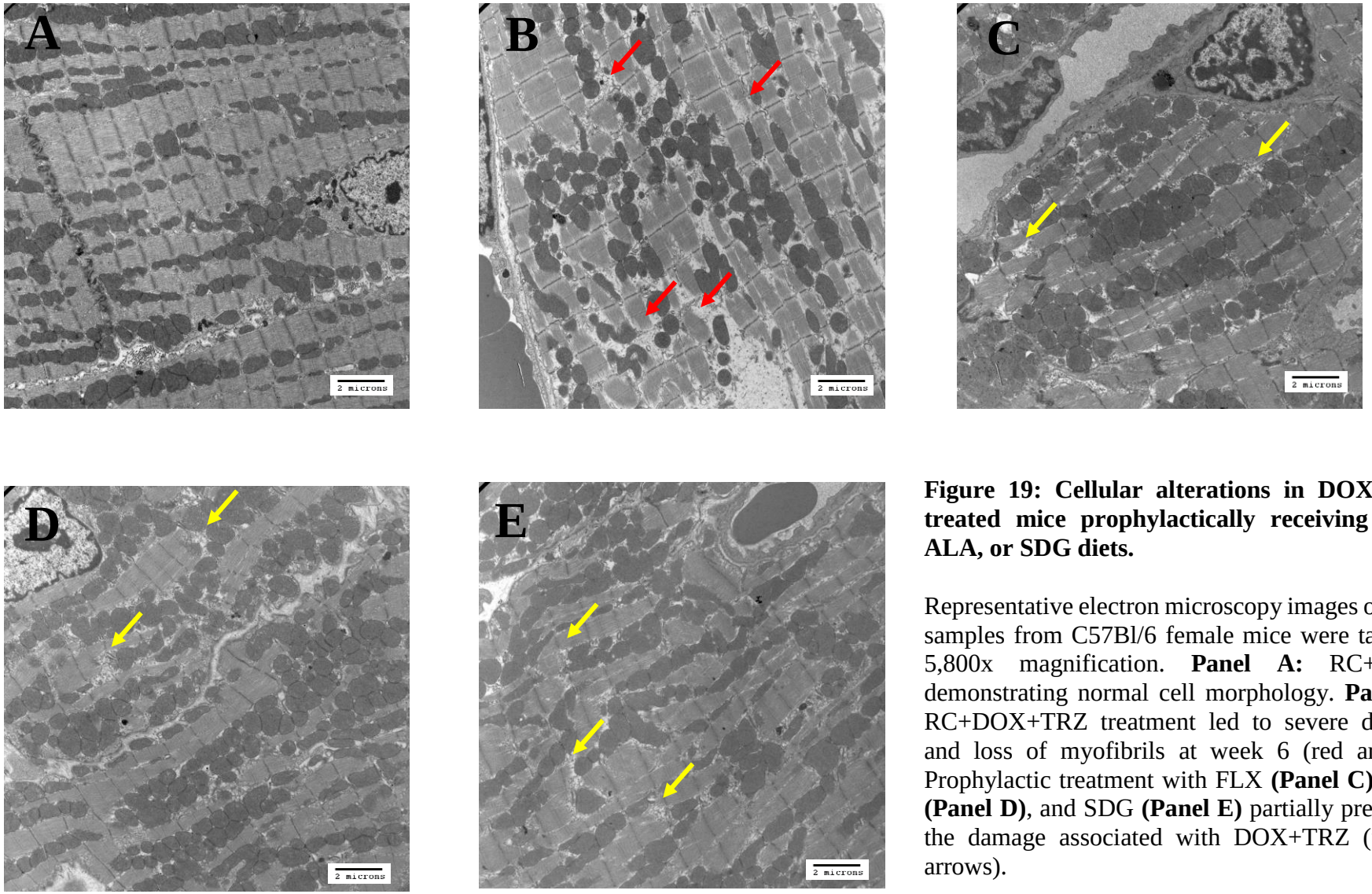


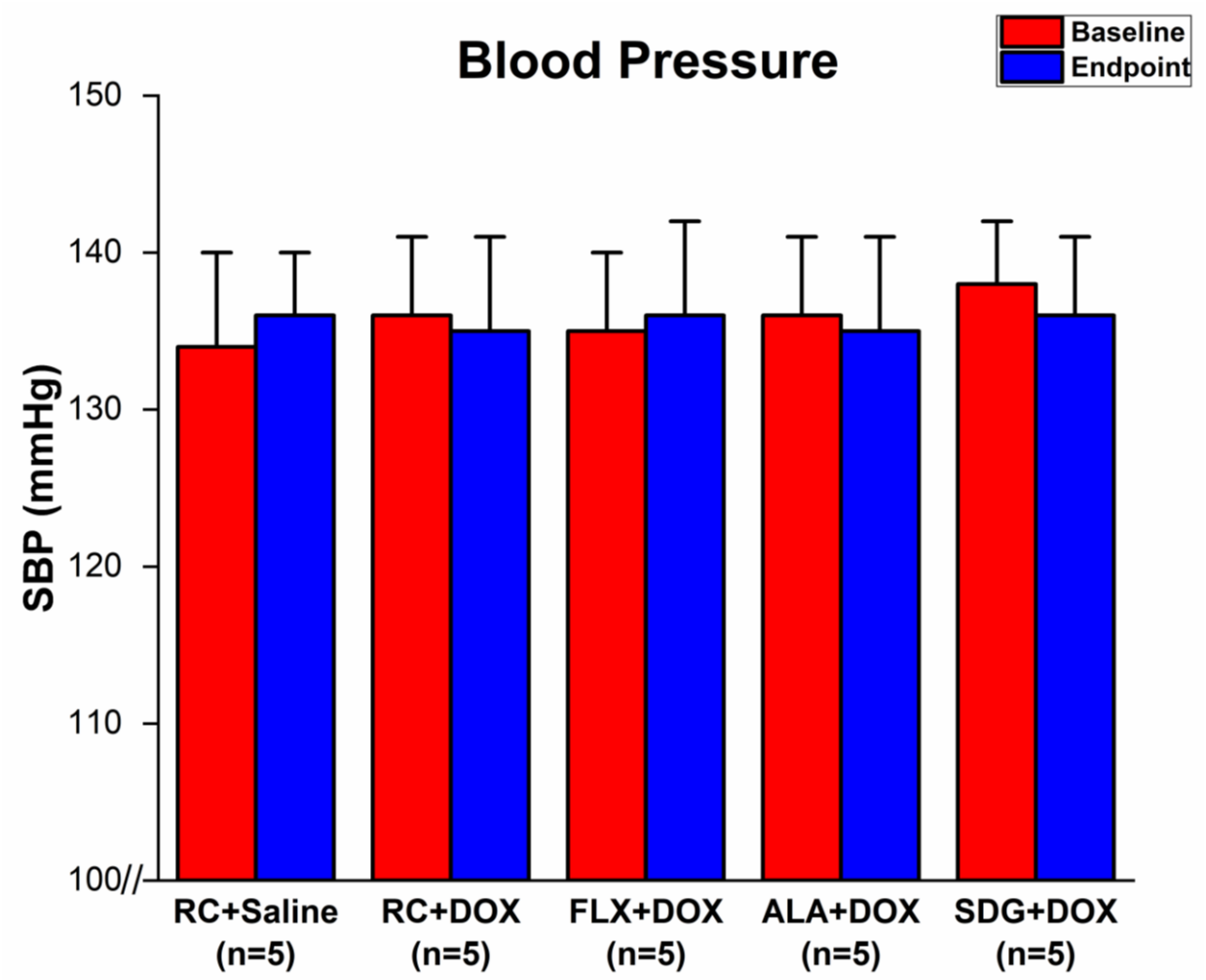
Figure 19: Cellular alterations in DOX+TRZ treated mice prophylactically receiving FLX, ALA, or SDG diets.

Representative electron microscopy images of heart samples from C57Bl/6 female mice were taken at 5,800x magnification. **Panel A:** RC+Saline demonstrating normal cell morphology. **Panel B:** RC+DOX+TRZ treatment led to severe damage and loss of myofibrils at week 6 (red arrows). Prophylactic treatment with FLX **(Panel C)**, ALA **(Panel D)**, and SDG **(Panel E)** partially prevented the damage associated with DOX+TRZ (yellow arrows).

Hemodynamics: DOX Treatment

Compared to baseline, the SBP of the mice that were treated with RC+Saline remained unchanged at week 6 (134±6 mmHg vs. 136±4 mmHg, respectively p=NS). Similarly, no significant differences in SBP were detected in any of the mice that were treated with DOX. Prophylactic administration of FLX, ALA, or SDG did not significantly alter SBP (Figure 20).

Figure 20: Changes in SBP in DOX treated mice receiving prophylactic treatment with FLX, ALA, or SDG.

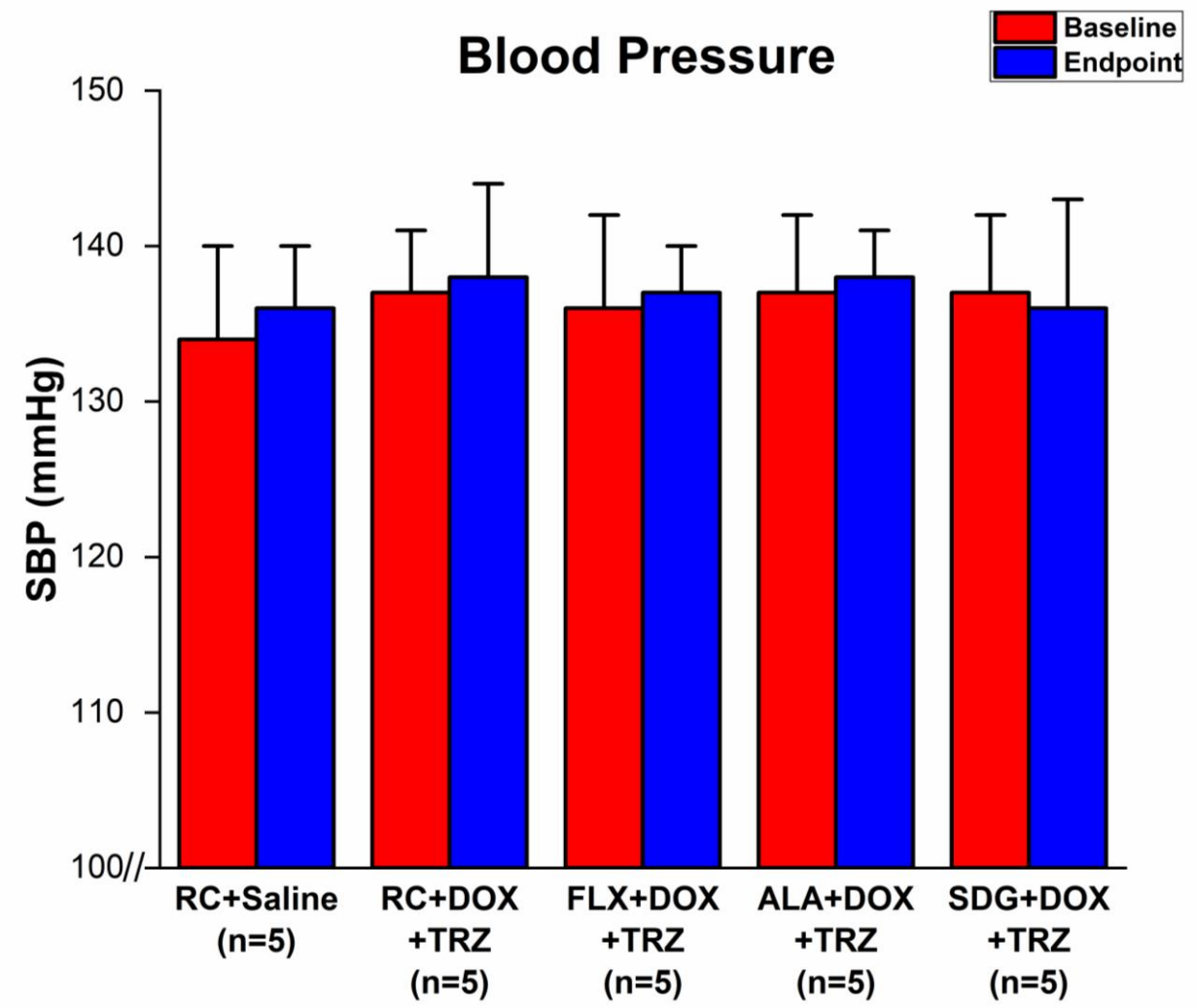


No significant changes in SBP were observed in C57Bl/6 female mice who were treated with RC+DOX at week 6 of the study. The prophylactic administration of FLX, ALA, and SDG in animals receiving DOX did not significantly alter SBP. The results are reported as mean ± SD. *p<0.05 RC+Saline vs. RC+DOX+TRZ. #p<0.05 RC+DOX+TRZ vs. FLX+DOX+TRZ, ALA+DOX+TRZ, or SDG+DOX+TRZ. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; RC, Regular Chow; SBP, Systolic Blood Pressure; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside.

Hemodynamics: DOX+TRZ Treatment

As compared to baseline, the SBP of the mice treated with RC+Saline remained unchanged at week 6 (134±6 mmHg vs. 136±4 mmHg, respectively p=NS). Similarly, no significant differences in SBP were detected in any of the mice that were treated with the combination of DOX+TRZ. Prophylactic administration of FLX, ALA, or SDG did not significantly alter SBP (Figure 21).

Figure 21: Changes in SBP in DOX+TRZ treated mice receiving prophylactic treatment with FLX, ALA, or SDG.

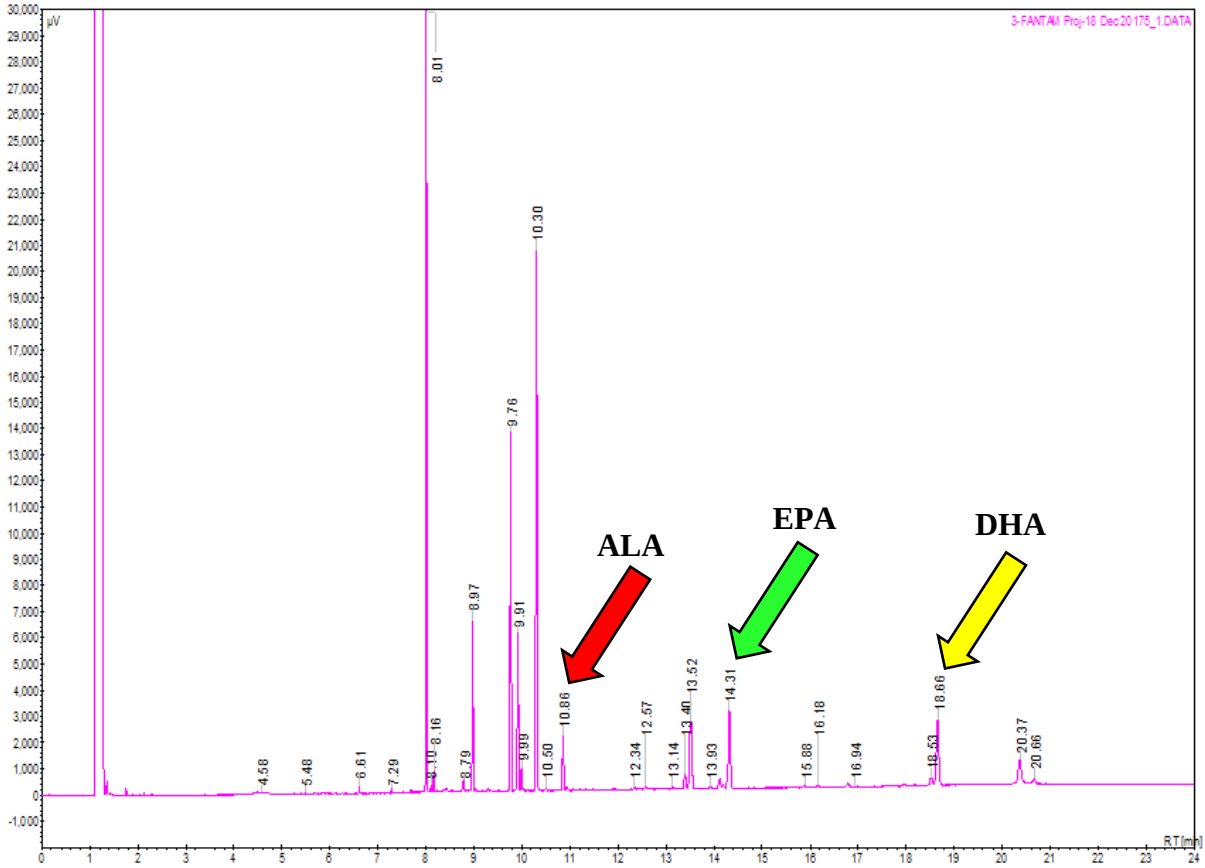


No significant changes in SBP were observed in C57Bl/6 female mice who were treated with RC+DOX+TRZ at week 6 of the study. The prophylactic administration of FLX, ALA, and SDG in animals receiving DOX+TRZ did not significantly alter SBP. The results are reported as mean ± SD. *$p<0.05$ RC+Saline vs. RC+DOX+TRZ. #$p<0.05$ RC+DOX+TRZ vs. FLX+DOX+TRZ, ALA+DOX+TRZ, or SDG+DOX+TRZ. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; RC, Regular Chow; SBP, Systolic Blood Pressure; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside; TRZ, Trastuzumab.

Chromatographic Identification of Fatty Acids

Gas chromatography and flame ionization detection was utilized to measure the plasma content of ALA and its downstream metabolites Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) in the RC, FLX, ALA, and SDG diet groups. Figure 22 denotes the complete fatty acid profile of the mouse plasma.

Figure 22: Representative fatty acid profile of the mouse plasma.



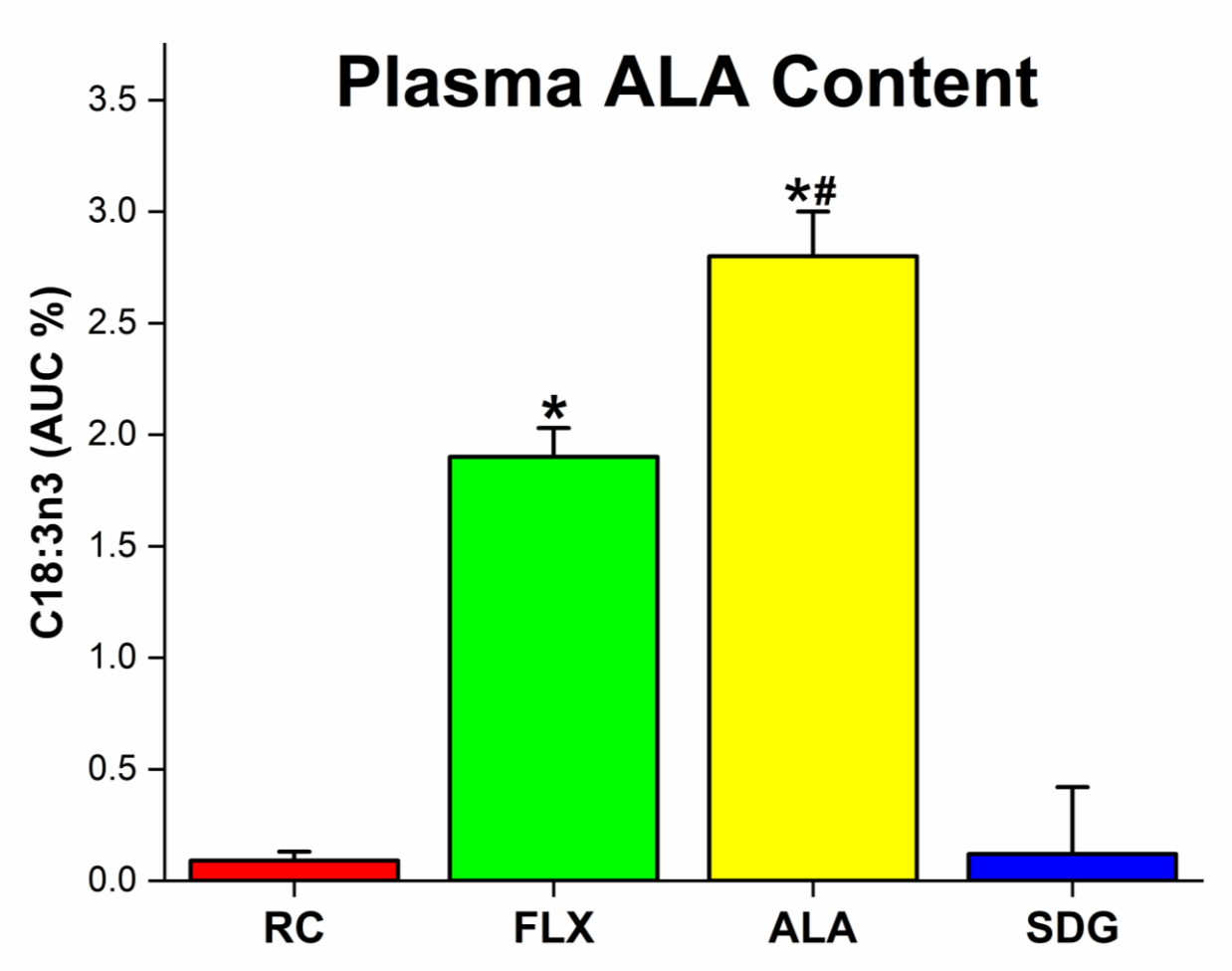
Red arrow: C18:3n3 fatty acid (ALA); green arrow: C20:5 fatty acid (EPA); yellow arrow: C22:6n3 fatty acid (DHA). ALA, Alpha-Linolenic Acid; DHA, Docosahexaenoic Acid; EPA, Eicosapentaenoic Acid.

The areas under the curve of the 10.86 peak, which represents ALA were quantified in each dietary group (Figure 23). Mice in the RC and SDG diet groups contained trace amounts of ALA with 0.14±0.04% and 0.12±0.30%, respectively. However, in the FLX and ALA diet groups, these values increased significantly to 1.89±0.13% and 2.79±0.20%, respectively ($p<0.05$).

The areas under the curve of the 14.31 peak, which represents the fatty acid EPA were quantified in each dietary group (Figure 24). Mice in the RC and SDG diet groups contained low levels of EPA with 0.61±0.18% and 1.24±0.44%, respectively. However, in the FLX and ALA diet groups, these values increased significantly to 3.01±1.23% and 3.55±1.18%, respectively ($p<0.05$).

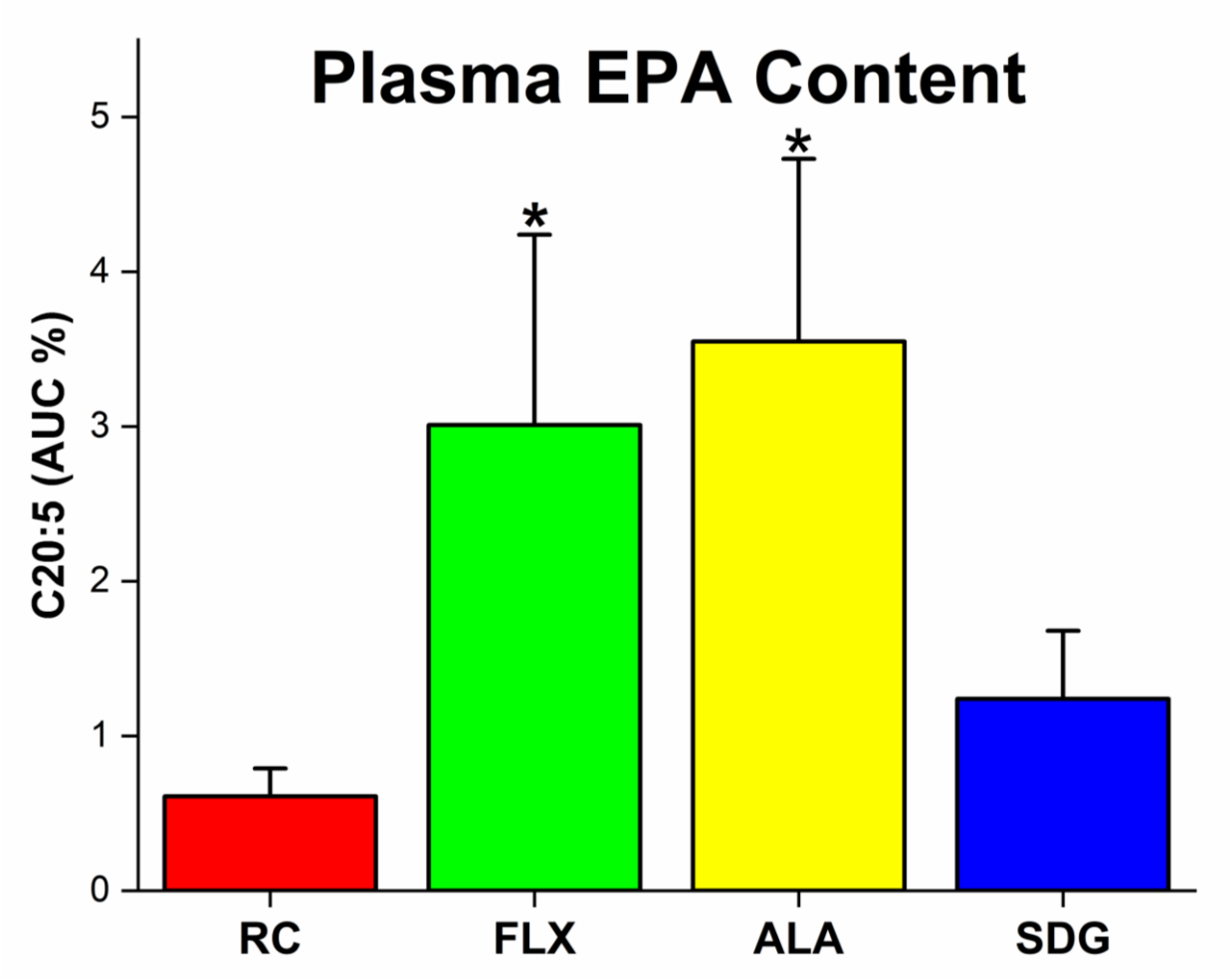
Finally, we quantified the areas under the curve of the 18.66 peak, which represents the fatty acid DHA were quantified in each dietary group (Figure 25). The levels of DHA in the RC, FLX, ALA, and SDG groups were not significantly different from each other with values of 4.38±1.32%, 4.69±1.66%, 4.37±1.46%, and 4.87±1.62%, respectively (p=NS). GC-FID analysis of the plasma was able to confirm the absorption of the study diets.

Figure 23: Circulating ALA plasma levels in treated mice prophylactically receiving FLX, ALA, or SDG.



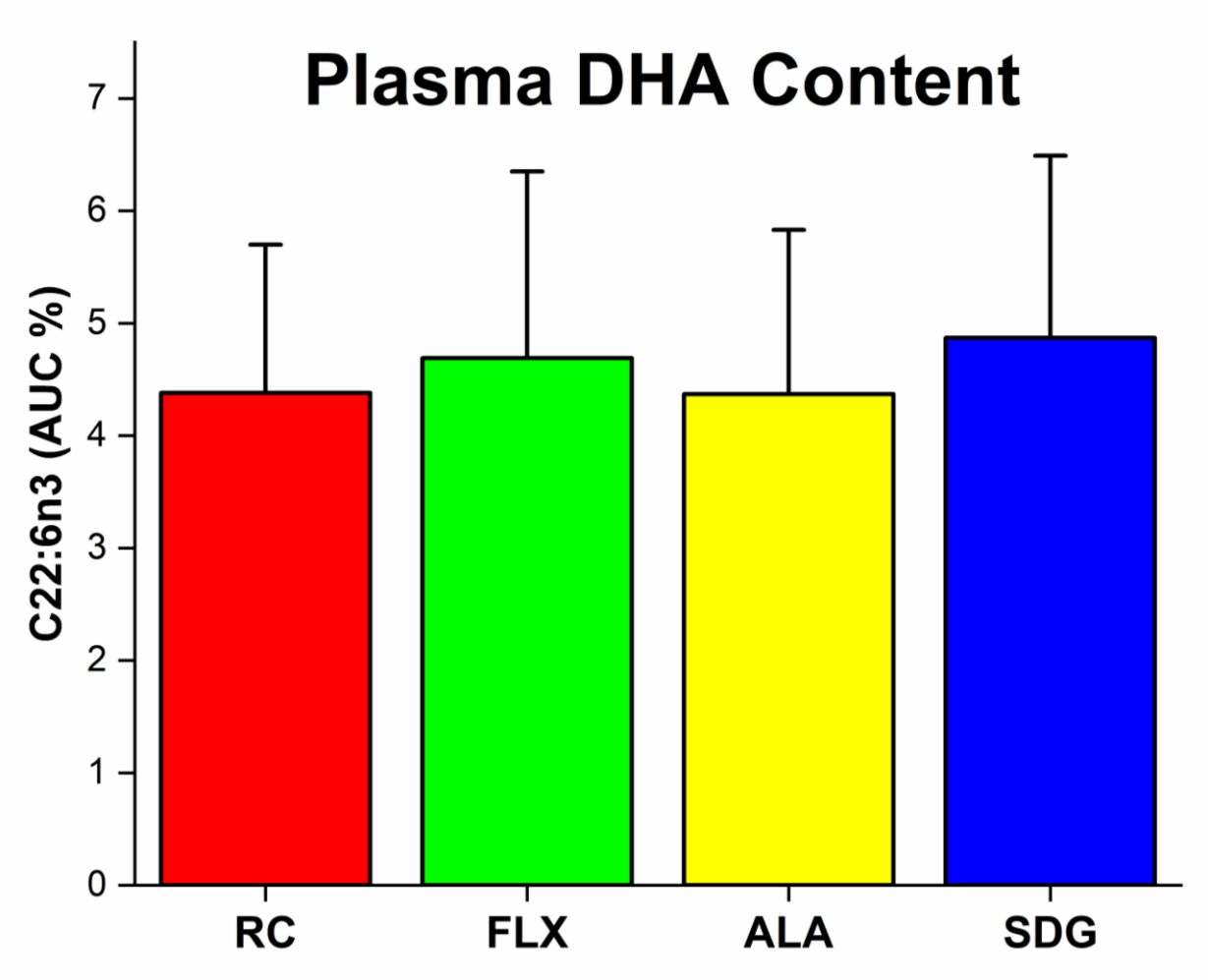
C57Bl/6 female mice treated with RC, FLX, ALA, or SDG. Plasma ALA values significantly increased in the mice who received prophylactic administration of the FLX and ALA study diets. The results are reported as mean ± SD. *p<0.05 RC and SDG vs. FLX. #p<0.05 FLX vs. ALA. ALA, Alpha-Linolenic Acid; AUC, Area Under the Curve; FLX, Flaxseed; RC, Regular Chow; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside.

Figure 24: Circulating EPA plasma levels in treated mice prophylactically recieving FLX, ALA, or SDG.



C57Bl/6 female mice treated with RC, FLX, ALA, and SDG. Plasma EPA values significantly increased in the mice who received prophylactic administration of the FLX and ALA study diets. The results are reported as mean ± SD. *$p<0.05$ RC and SDG vs. FLX and ALA. ALA, Alpha-Linolenic Acid; AUC, Area Under the Curve; EPA, Eicosapentaenoic Acid; FLX, Flaxseed; RC, Regular Chow; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside.

Figure 25: Circulating DHA plasma levels in treated mice prophylactically receiving FLX, ALA, or SDG.

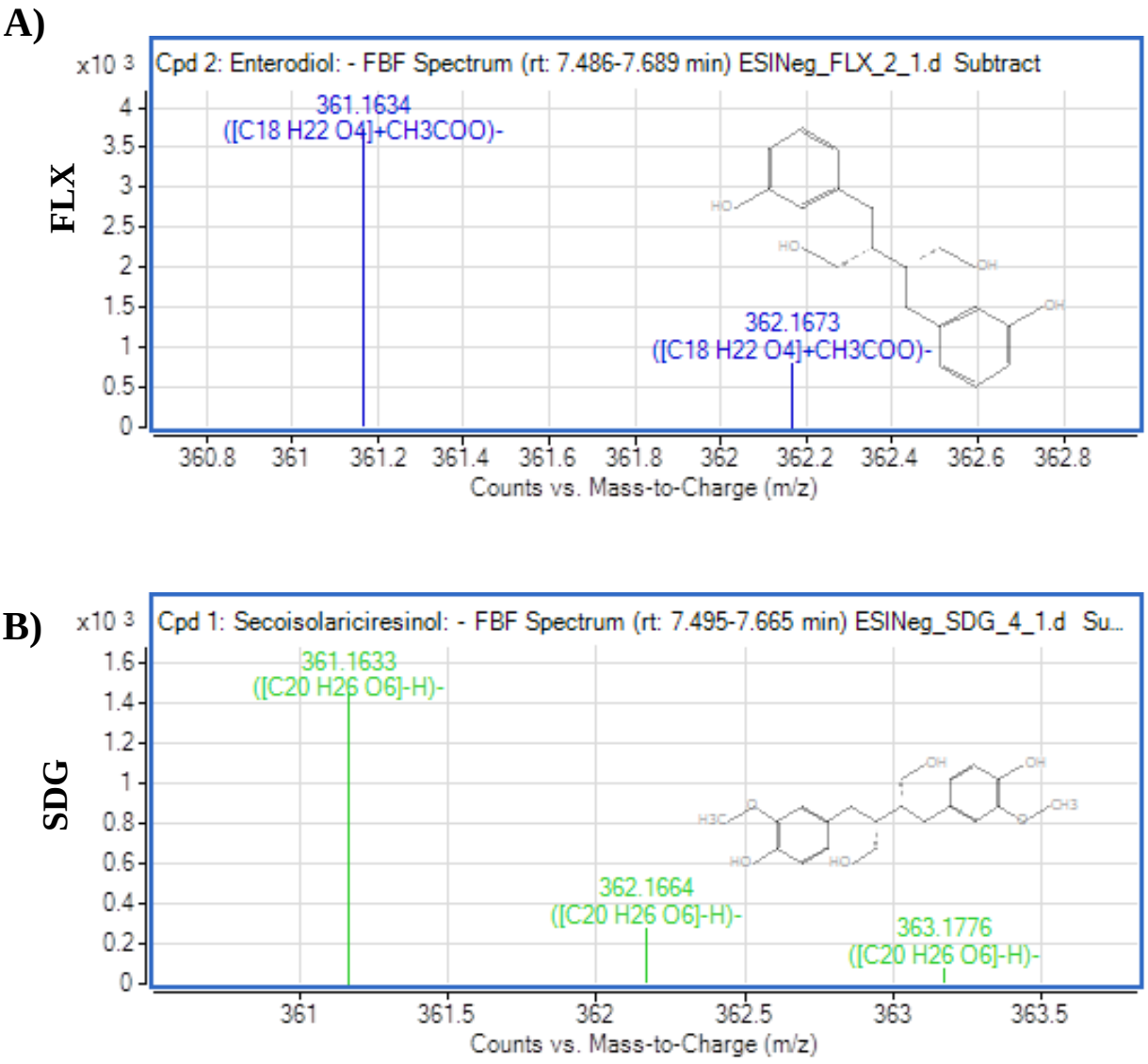


C57Bl/6 female mice treated with RC, FLX, ALA, and SDG. Plasma DHA values did not significantly change in the mice who received prophylactic administration with any of the study diets. The results are reported as mean ± SD. ALA, Alpha-Linolenic Acid; AUC, Area Under the Curve; DHA, Docosahexaenoic Acid; FLX, Flaxseed; RC, Regular Chow; SD, Standard Deviation; SDG, Secoisolariciresinol Diglucoside.

Lignan Metabolite Content

Quadrupole time-of-flight mass spectroscopy (Q-TOF-MS) was utilized to identify SDG metabolites in the RC+Saline, FLX+Saline, ALA+Saline, and SDG+Saline treatment groups. The SDG metabolite Enterodiol was identified in the FLX+Saline treatment group only (Figure 26A). Similarly, the SDG metabolite Secoisolariciresinol was only identified in the mice treated with SDG+Saline (Figure 26B). No SDG metabolites were detected in the RC+Saline or ALA+Saline treated mice (data not shown). Q-TOF-MS analysis of the plasma confirms absorption of the diets.

Figure 26: Identified SDG metabolites.



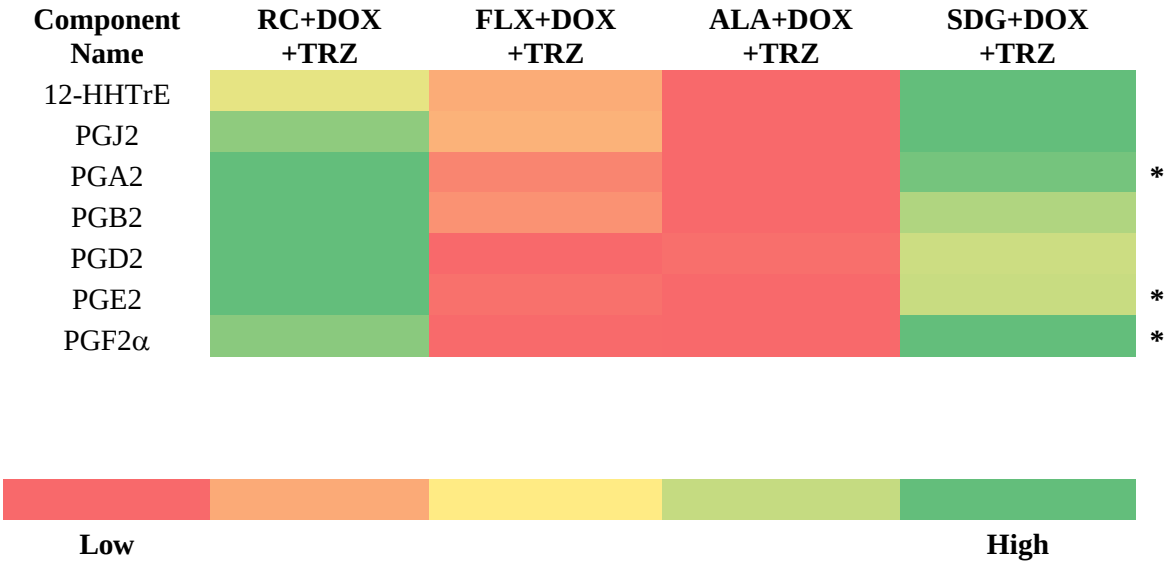
A) Enterodiol detected in the FLX+Saline treated mice. **B)** Secoisolariciresinol detected in the SDG+Saline treated mice. FLX, Flaxseed; SDG, Secoisolariciresinol Diglucoside.

Changes in Plasma Oxylipin Concentrations

HPLC ESI-MS was used to quantify circulating levels of plasma oxylipins from the various treatment groups. In mice treated with RC+DOX+TRZ, there was a significant increase the in concentration of COX-derived oxylipins ($p<0.05$) (Figure 27). Interestingly, the prophylactic administration of the FLX and ALA diets significantly lowered the concentration of these inflammatory oxylipins ($p<0.05$) (Figure 27).

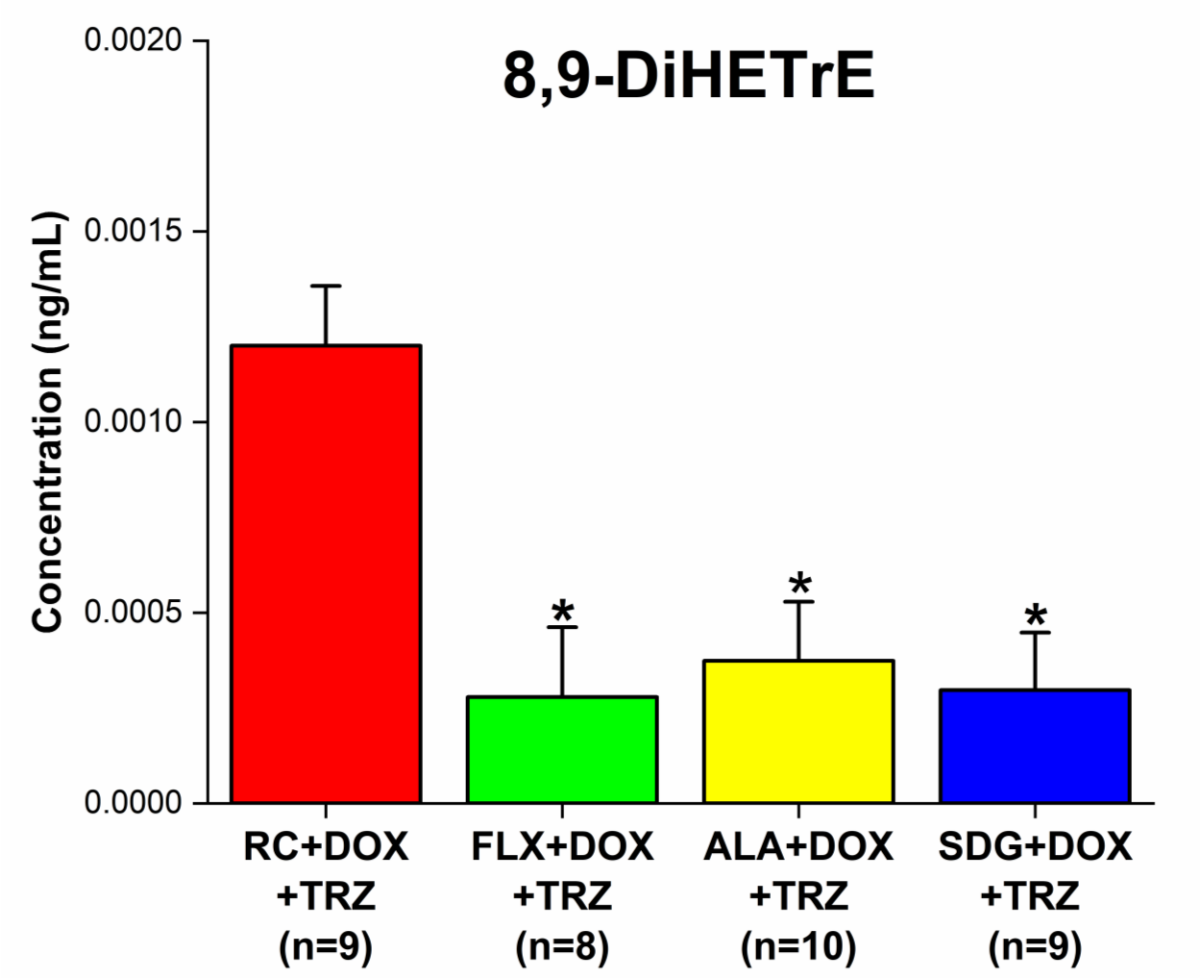
Additionally, there was a significant increase in 8,9-Dihydroxyeicosatrienoic acid (8,9-DiHETrE) concentration in the RC+DOX+TRZ treated mice ($p<0.05$) (Figure 28). However, the prophylactic administration of FLX, ALA, or SDG was able to decrease the concentration of this apoptotic marker ($p<0.05$) (Figure 28).

Figure 27: Heat map representing the changes in concentration of COX-derived oxylipins in DOX+TRZ treated mice prophylactically receiving FLX, ALA, or SDG diets.



Analysis of the plasma from C57Bl/6 female mice treated with RC+DOX+TRZ with prophylactic FLX, ALA, or SDG diets at week 6. Treatment with RC+DOX+TRZ increased the concentration of COX-derived oxylipins. The prophylactic treatment with FLX, ALA, or SDG normalized the concentration of these inflammatory markers. The results are reported as mean. *$p<0.05$ RC+DOX+TRZ vs. FLX+DOX+TRZ or ALA+DOX+TRZ. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; PGA_2, Prostaglandin A_2; PGB_2, Prostaglandin B_2; PGD_2, Prostaglandin D_2; PGE_2, Prostaglandin E_2; $PGF_{2\alpha}$, Prostaglandin $F_{2\ alpha}$; PGJ_2, Prostaglandin J_2; RC, Regular Chow; SDG, Secoisolariciresinol Diglucoside; TRZ, Trastuzumab; 12-HHTrE, 12-Hydroxyheptadecatrienoic acid.

Figure 28: Changes in 8,9-DiHETrE concentration in DOX+TRZ treated mice prophylactically receiving FLX, ALA, or SDG diets.



Analysis of the plasma from C57Bl/6 female mice treated with DOX+TRZ with prophylactic FLX, ALA, or SDG diets at week 6. Treatment with RC+DOX+TRZ increased 8,9-DiHETrE concentration. The prophylactic treatment with FLX, ALA, or SDG normalized the concentration of this inflammatory marker. The results are reported as mean ± SEM. *$p<0.05$ RC+DOX+TRZ vs. FLX+DOX+TRZ, ALA+DOX+TRZ, or SDG+DOX+TRZ. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; RC, Regular Chow; SDG, Secoisolariciresinol Diglucoside; SEM, Standard Error Mean; TRZ, Trastuzumab; 8,9-DiHETrE, 8,9-Dihydroxyeicosatrienoic acid.

Western Blotting: DOX+TRZ Treatment

In mice treated with RC+DOX+TRZ, there was a 2.0-fold increase in NF-κB expression as compared to the RC+Saline control ($p<0.05$) (Figure 29A-B). Interestingly, the prophylactic administration of FLX, ALA, or SDG significantly down-regulated the expression of this inflammatory marker ($p<0.05$) (Figure 29A-B).

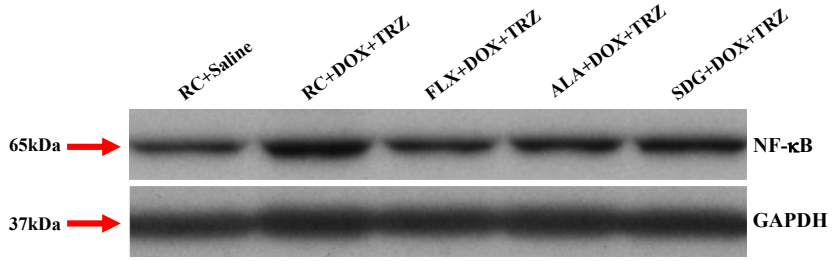
In mice receiving RC+DOX+TRZ, the expression of the pro-apoptotic protein PARP increased 1.8-fold, as compared to RC+Saline ($p<0.05$) (Figure 30A-B). However, the prophylactic administration of FLX, ALA, or SDG mitigated this increase in PARP expression ($p<0.05$) (Figure 30A-B). Additionally, the Bax/Bcl-X_L ratio significantly increased 1.8-fold in RC+DOX+TRZ treated mice, as compared to control ($p<0.05$) (Figure 31A-B). The prophylactic administration of FLX, ALA, or SDG significantly attenuated this increase ($p<0.05$) (Figure 31A-B).

Finally, there was a 1.9-fold increase in Bnip3 expression in the RC+DOX+TRZ treated mice, as compared to the RC+Saline control group ($p<0.05$) (Figure 32A-B). Prophylactic treatment with FLX, ALA or SDG prevented this increase in Bnip3 expression ($p<0.05$) (Figure 32A-B).

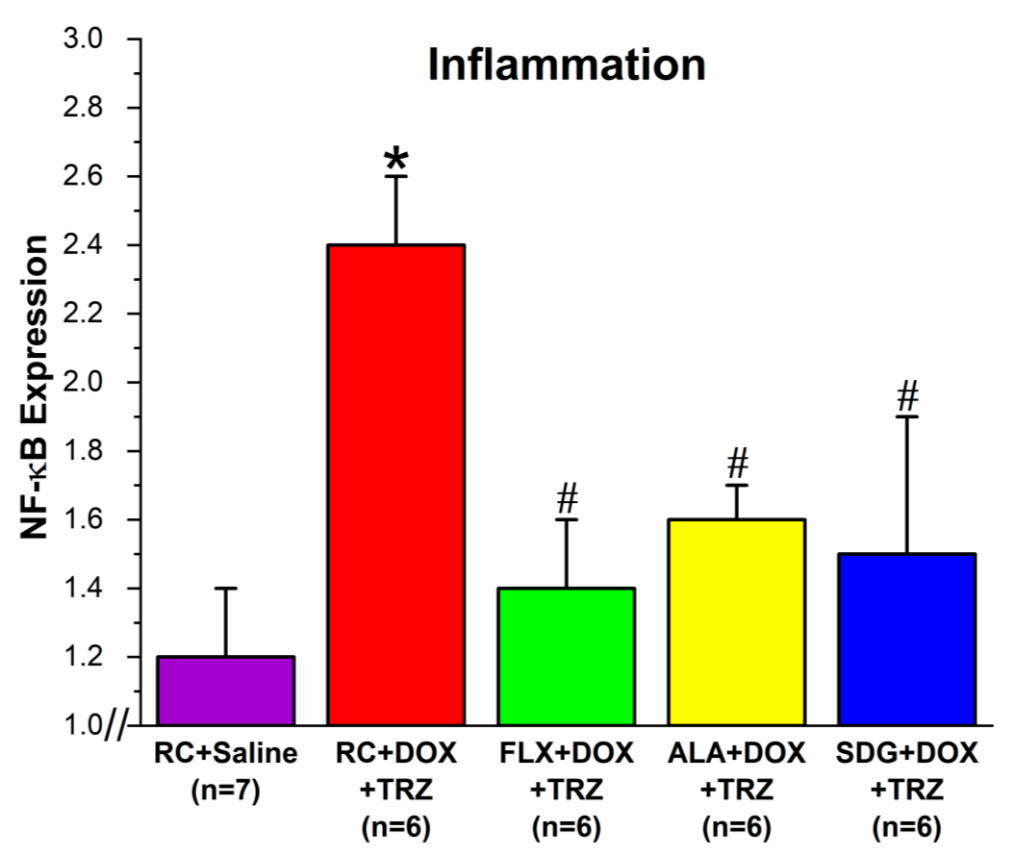
Expressions of other markers including Caspase-3, TNF-α, IL-1β, IL-6, and PPAR-α were assessed at week 6. No significant changes were observed between the various treatment groups for these markers (data not shown).

Figure 29: Changes in NF-κB expression in DOX+TRZ treated mice prophylactically receiving FLX, ALA, or SDG diets.

A)



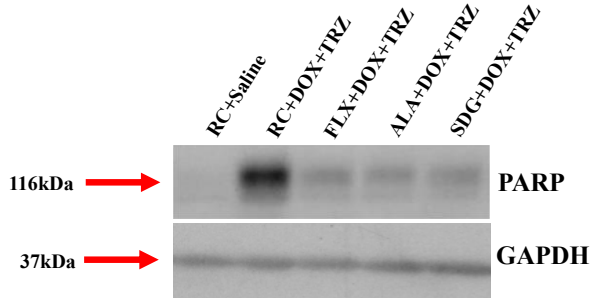
B)



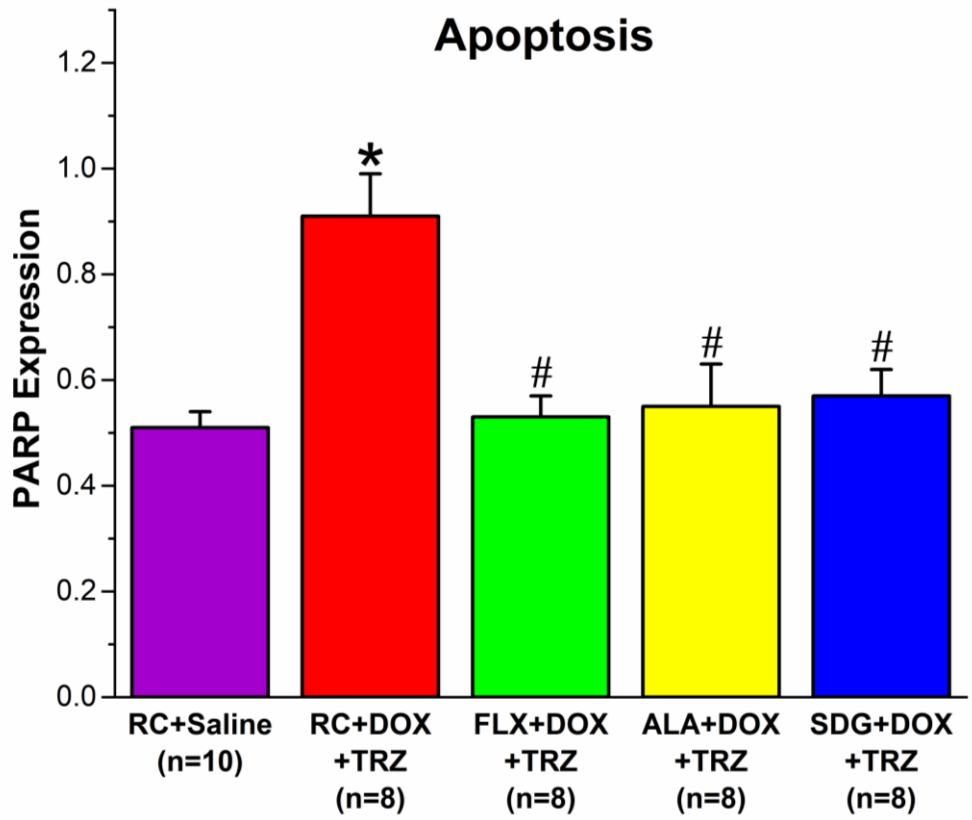
A) Representative Western blot. **B)** Data as fold change of NF-κB expression in C57Bl/6 female mice treated with saline or DOX+TRZ receiving daily prophylactic treatment with FLX, ALA, or SDG. Treatment with RC+DOX+TRZ significantly up-regulated NF-κB expression. The prophylactic treatment with FLX, ALA, or SDG normalized the expression of this inflammatory marker. The results are normalized to GAPDH loading control and are reported as mean ± SEM. *p<0.05 RC+Saline vs. RC+DOX+TRZ. #p<0.05 RC+DOX+TRZ vs. FLX+DOX+TRZ, ALA+DOX+TRZ, or SDG+DOX+TRZ. The results are reported as mean ± SEM. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; NF-κB, Nuclear Factor Kappa B; RC, Regular Chow; SDG, Secoisolariciresinol Diglucoside; SEM, Standard Error Mean; TRZ, Trastuzumab.

Figure 30: Changes in PARP expression in DOX+TRZ treated mice prophylactically receiving FLX, ALA, or SDG diets.

A)



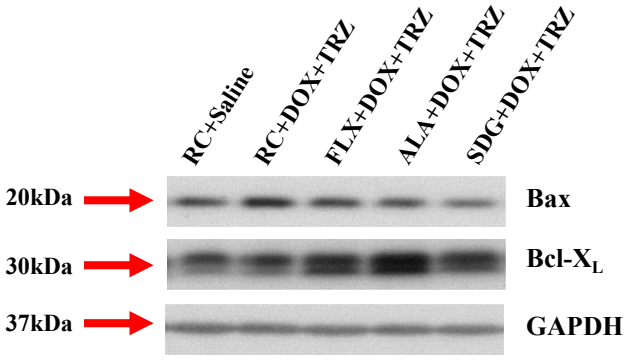
B)



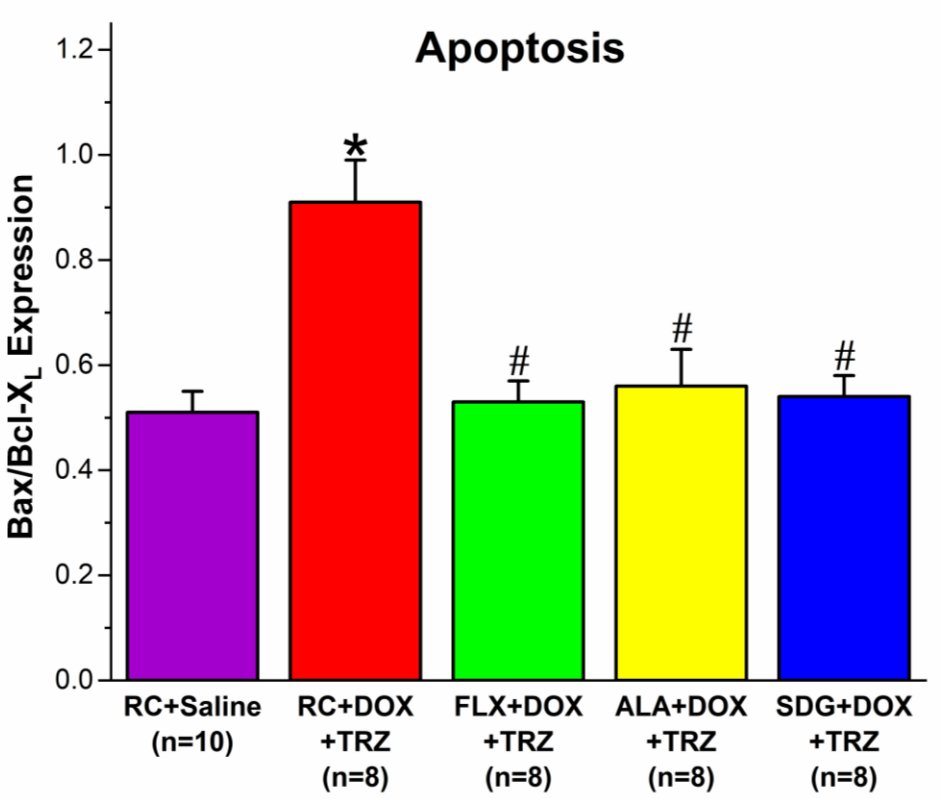
A) Representative Western blot. **B)** Data as fold change of PARP expression in C57Bl/6 female mice treated with saline or DOX+TRZ receiving daily prophylactic treatment with FLX, ALA, or SDG. Treatment with RC+DOX+TRZ significantly up-regulated PARP expression. The prophylactic treatment with FLX, ALA, or SDG normalized the expression of this apoptotic marker. The results are normalized to GAPDH loading control and are reported as mean ± SEM. *p<0.05 RC+Saline vs. RC+DOX+TRZ. #p<0.05 RC+DOX+TRZ vs. FLX+DOX+TRZ, ALA+DOX+TRZ, or SDG+DOX+TRZ. The results are reported as mean ± SEM. ALA, Alpha-Linolenic Acid; DOX, Doxorubicin; FLX, Flaxseed; PARP, Poly (ADP-ribose) polymerase; RC, Regular Chow; SDG, Secoisolariciresinol Diglucoside; SEM, Standard Error Mean; TRZ, Trastuzumab.

Figure 31: Changes in Bax/Bcl-X_L expression in DOX+TRZ treated mice prophylactically receiving FLX, ALA, or SDG diets.

A)



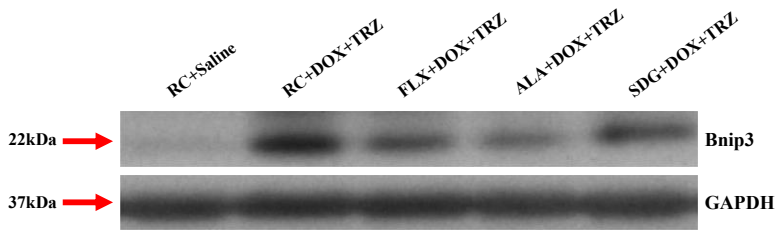
B)



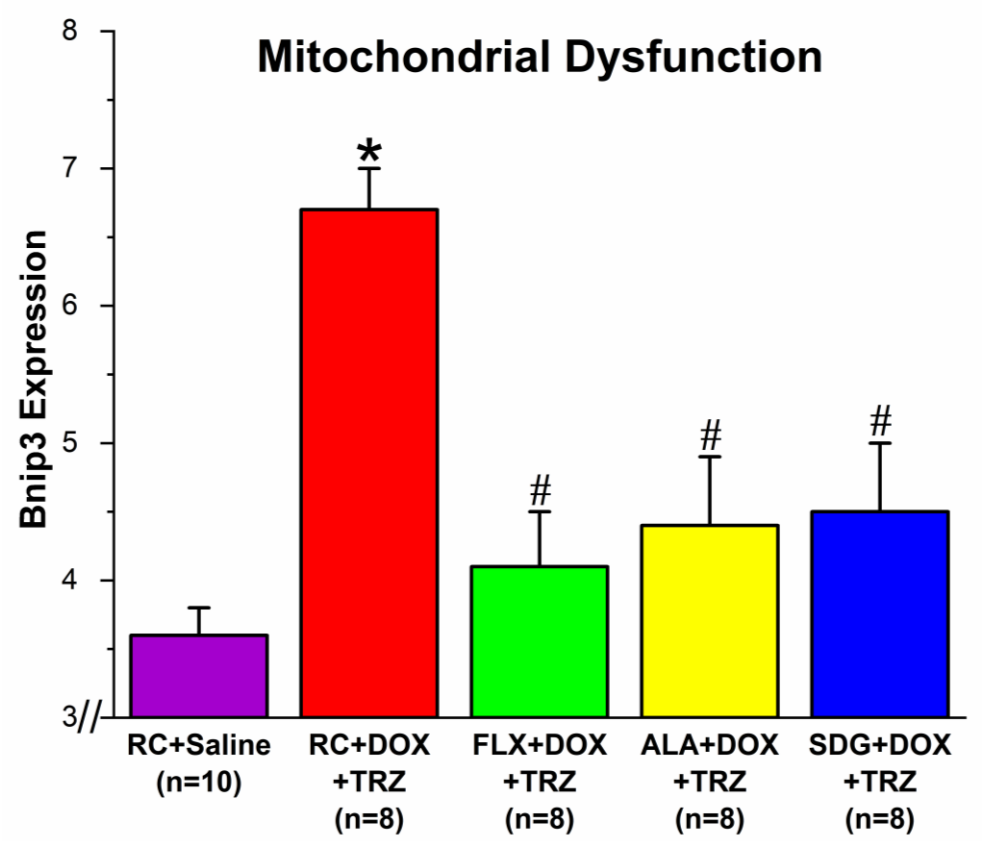
A) Representative Western blot. **B)** Data as fold change of Bax/Bcl-X_L expression in C57Bl/6 female mice treated with saline or DOX+TRZ receiving daily prophylactic treatment with FLX, ALA, or SDG. Treatment with RC+DOX+TRZ significantly up-regulated Bax/Bcl-X_L expression. The prophylactic treatment with FLX, ALA, or SDG normalized the expression of this apoptotic marker. The results are normalized to GAPDH loading control and are reported as mean ± SEM. *$p<0.05$ RC+Saline vs. RC+DOX+TRZ. #$p<0.05$ RC+DOX+TRZ vs. FLX+DOX+TRZ, ALA+DOX+TRZ, or SDG+DOX+TRZ. The results are reported as mean ± SEM. ALA, Alpha-Linolenic Acid; Bax, Bcl-2 associated X protein; Bcl-X_L, B-cell lymphoma extra-large; DOX, Doxorubicin; FLX, Flaxseed; RC, Regular Chow; SDG, Secoisolariciresinol Diglucoside; SEM, Standard Error Mean; TRZ, Trastuzumab.

Figure 32: Changes in Bnip3 expression in DOX+TRZ treated mice prophylactically receiving FLX, ALA, or SDG diets.

A)



B)



A) Representative Western blot. **B)** Data as fold change of Bnip3 expression in C57Bl/6 female mice treated with saline or DOX+TRZ receiving daily prophylactic treatment with FLX, ALA, or SDG. Treatment with RC+DOX+TRZ significantly up-regulated Bnip3 expression. The prophylactic treatment with FLX, ALA, or SDG normalized the expression of this marker of mitochondrial dysfunction. The results are normalized to GAPDH loading control and are reported as mean ± SEM. *$p<0.05$ RC+Saline vs. RC+DOX+TRZ. #$p<0.05$ RC+DOX+TRZ vs. FLX+DOX+TRZ, ALA+DOX+TRZ, or SDG+DOX+TRZ. The results are reported as mean ± SEM. ALA, Alpha-Linolenic Acid; Bnip3, Bcl-2 interacting protein 3; DOX, Doxorubicin; FLX, Flaxseed; RC, Regular Chow; SDG, Secoisolariciresinol Diglucoside; SEM, Standard Error Mean; TRZ, Trastuzumab.

Chapter 6: Discussion

6.1 Overall Summary

New developments in cancer treatment including chemotherapy, radiation, endocrine, and targeted therapies have resulted in cancer patients living longer. However, chemotherapy induced cardiovascular disease remains a serious concern as it is a cause of short- and long-term morbidity and mortality in this patient population. The evolving field of Cardio-Oncology is critical for the development of improved survivorship strategies after the successful application of oncologic therapies to improve patient life expectancy.[177] Cardio-Oncology has led to advancements in the prevention, diagnosis, and management of cancer patients who develop cardiovascular complications as a result of their cancer treatment.

Our study demonstrated for the first time that the prophylactic treatment with FLX, ALA, or SDG significantly attenuated the cardiotoxic side effects of the combination therapy of DOX+TRZ in a chronic *in vivo* female murine model of chemotherapy induced cardiomyopathy. Specifically, we demonstrated that FLX: i) prevents adverse left ventricular remodeling due to DOX+TRZ; ii) diminishes myofibrillar disarray; iii) reduces the degree of inflammation following treatment with DOX+TRZ; iv) and attenuates the degree of cardiac apoptosis, OS, and mitochondrial dysfunction.

6.2 Cardiovascular Remodeling

Serial monitoring of LVEF using non-invasive cardiac imaging techniques is the single most important diagnostic tool used in clinical practice to detect chemotherapy induced cardiac dysfunction. Unfortunately, irreversible cardiac damage may occur once heart failure in the setting of a reduced LVEF develops.[65, 92] Several basic science studies in the field of Cardio-Oncology

have evaluated the role of cardiac imaging for the early detection of LV systolic dysfunction following treatment with DOX+TRZ.[394]

In a study conducted by Neilan *et al.* (2006), the authors investigated whether or not TDI could predict left ventricular systolic dysfunction (LVSD) and mortality in an acute *in vivo* murine model of DOX induced cardiotoxicity.[381] In this study, a single dose of DOX (20 mg/kg) was administered to C57Bl/6 mice and serial evaluation of TDI-derived peak endocardial systolic velocity (V_{ENDO}), stain rate (SR), as well as M-mode and 2D indices of LV systolic function was performed for 5 days.[381] As compared to saline control mice, left ventricular fractional shortening (LVFS) and LVEF did not change until 5 days after the administration of DOX where both of these parameters decreased by 10±1% ($p<0.001$ and $p=0.01$, respectively).[381] Although conventional echocardiographic indices remained unchanged after the acute administration of DOX, V_{ENDO} and SR as assessed by TDI decreased significantly by 24hrs and 48hrs post-injection, respectively ($p<0.05$).[381] This study demonstrated that TDI and strain rate parameters on echocardiography can detect early evidence of LVSD as compared to conventional echo parameters (LVFS and LVEF) in a murine model.

Neilan *et al.* (2006), also carried out a chronic *in vivo* study of DOX induced cardiotoxicity using a mouse model.[381] In this study, mice were treated with serial injections of DOX (4 mg/kg weekly for 5 weeks) to mimic the clinical setting.[381] This study concluded that mice treated with DOX developed LV cavity dilatation with a LVEDD of 3.2±0.1 mm at baseline, which increased to 4.2±0.2 mm by week 16 ($p<0.05$).[381] The LVEF decreased significantly from 78±1% at baseline to 42±2% at week 16 ($p<0.05$).[381] However, TDI parameters detected the development of LVSD

6 weeks earlier than conventional indices (LVFS and LVEF).[381] Specifically, the V_{ENDO} significantly decreased from 3.3±0.1 cm/s at baseline to 2.4±0.1 cm/s by week 6 of the study, detecting early LV dysfunction as compared to LVFS and LVEF ($p<0.05$). These conventional indices did not significantly decrease until the end of the 16-week study.[381] Specifically, LVFS decreased from 57±1% at baseline to 28±1% by week 16 of the study, and LVEF decreased from 78±1% at baseline to 42±2% at week 16 ($p<0.05$).[381] This comprehensive study by Neilan *et al.* (2006) demonstrated that TDI detects LV dysfunction prior to changes in conventional echocardiographic indices as well as predicts increased mortality.[381] Therefore, it is suggested that TDI may be a reliable tool to detect early subclinical changes in DOX induced cardiotoxicity.

In a separate study by Neilan *et al.* (2007), both the acute and chronic cardiotoxic side effects of DOX were evaluated in an *in vivo* murine model of DOX induced cardiotoxicity.[394] Specifically, they investigated the role of endothelial nitric oxide synthase [nitric oxide synthase 3 (NOS3)] in cardiac dysfunction and injury that is typically observed after the administration of DOX.[394] In the acute portion of the study, a single dose of DOX (20 mg/kg) was administered to WT mice, NOS3-deficient mice ($NOS3^{-/-}$), and mice with cardiomyocyte specific overexpression of NOS3 (NOS3-TG).[394] Cardiac function was assess using echocardiography 5 days post injection.[394] In WT mice receiving DOX, LVFS decreased from 57±2% at baseline to 47±1% at day 5 ($p<0.001$).[394] Comparatively, LVFS was greater in $NOS3^{-/-}$ mice and reduced in NOS3-TG mice after the administration of DOX with LVFS values of 55±1% and 35±2%, respectively ($p=0.56$ and $p<0.001$).[394] A similar trend was observed for the LVEF parameters. Specifically, LVEF decreased from 78±2% at baseline to 66±3% at day 5 in WT mice ($p<0.001$).[394] In comparison, LVEF was

greater in NOS3$^{-/-}$ mice and reduced in NOS3-TG mice after the administration of DOX with LVEF values of 74±2% and 52±3%, respectively ($p=0.32$ and $p<0.001$).[394]

In 2009, Jassal *et al.* expanded on these findings by studying the combined cardiotoxic side effects of DOX (20 mg/kg), TRZ (10 mg/kg), and liposomal encapsulated DOX (Myocet; 10 mg/kg) in an acute *in vivo* murine model of cardiotoxicity.[57] The purpose of this study was to determine if TDI is useful for the early detection of DOX+TRZ mediated LV dysfunction. In this study, serial echocardiographic assessments were performed on awake mice for 5 days.[57] Progressive LV dilatation and LVSD were observed in mice treated with DOX+TRZ by day 4 of the study.[57] Specifically, mice that were treated with DOX+TRZ had a significant decrease in LVEF from 75±2% at baseline to 63±2% by day 4 ($p<0.05$).[57] Comparatively, LVEF was preserved in the remaining study groups. However, TDI parameters decreased significantly within 24 hours of drug administration in the mice that were treated with either DOX by itself or the combination of DOX+TRZ and predicted early mortality.[57]

More recently, a study by Walker *et al.* (2011) reported that in mice treated with DOX (20 mg/kg), LVEDD significantly increased from 3.2±0.1 mm at baseline to 3.8±0.2 mm by day 10 of the study.[54] Furthermore, the combined treatment of DOX+TRZ (20 mg/kg; 10 mg/kg) resulted in a significant increase in LVEDD from 3.1±0.1 mm at baseline to 4.1±0.2 mm by day 10 of the study.[54] Moreover, the prophylactic treatment with Probucol, an anti-oxidant, demonstrated a cardioprotective effect by attenuating this increase in LVEDD. Specifically, LVEDD only increased from 3.1±0.1 mm at baseline to 3.6±0.1 mm by day 10 of the study.[54] This study also evaluated sensitive indices of LV dysfunction. Specifically, V_{ENDO} and SR revealed that LVSD

had developed by day 10 of the study in mice treated with DOX or the combination of DOX+TRZ ($p<0.05$).[54] However, the administration of the anti-oxidant Probucol was cardioprotective as reflected by a decrease in TDI parameters, preserved cardiac function, and decrease mortality by 40%.[54]

In a follow-up study that was performed by Zeglinski *et al.* (2014), WT female mice in an acute *in vivo* model of chemotherapy mediated cardiotoxicity were treated with either DOX alone (20mg/kg) or the combination of DOX+TRZ (20 mg/kg; 10 mg/kg).[60] Serial echocardiographic assessment of these mice confirmed a decrease in LVEF from 72±3% at baseline to 35±2% by day 10 of the study ($p < 0.05$).[60] Similarly, LVEDD increased from 3.1±0.2 at baseline to 4.2±0.2 by the end of the 10-day study, demonstrating the development of LVSD.[60] Similar to previous studies, Zeglinski *et al.* (2014) also reported on the early indices of cardiotoxicity detected through the use of TDI. In mice treated with the combination of DOX+TRZ, V_{ENDO} significantly decreased from 3.3±0.2% at baseline to 1.2±0.2% by the end of the acute study period ($p<0.05$).[60]

Most recently, Goyal *et al.* (2016) published a study evaluating the role of the anti-oxidant N-acetylcysteine amide (NACA) in the prevention of DOX+TRZ mediated cardiotoxicity in the acute setting.[64] In this study, mice were treated with DOX (20 mg/kg) alone or the combination of DOX+TRZ (20 mg/kg; 10 mg/kg).[64] Echocardiographic assessments were performed on awake mice daily for 10 days.[64] In mice receiving treatment with DOX alone, LVEF significantly decreased from 73±4% at baseline to 43±2% by day 10 of the study ($p<0.05$).[64] Similarly, in the mice treated with the combination of DOX+TRZ, LVEF values significantly decreased from 72±3% at baseline to 32±2% on day 10 ($p<0.05$).[64] However, the prophylactic treatment with

NACA, an anti-oxidant, demonstrated a cardioprotective effect by partially attenuating this decrease in LVEF.[64] In WT mice treated with DOX alone, LVEDD significantly increased from 3.2±0.2 mm at baseline to 3.9±0.2 mm by day 10 of the 10-day study ($p<0.05$).[64] Similar findings were observed in mice who were being treated with the combination of DOX+TRZ as the LVEDD increased from 3.2±0.1 mm at baseline to 4.3±0.2 mm by day 10 of the study ($p<0.05$).[64] Once again, prophylactic treatment with NACA was able to prevent this increase in LVEDD in mice receiving either DOX alone or the combination treatment of DOX+TRZ.[64]

While the above-mentioned studies investigated the role of pharmacological anti-oxidant agents including Probucol and NACA in the prevention of chemotherapy induced cardiotoxicity, little is known on the cardioprotective role of nutraceuticals in this setting. Akolkar *et al.* (2017) investigated the effect of prophylactic treatment with Vitamin C (50 mg/kg) on DOX induced cardiotoxicity.[285] In this study, a cumulative dose of 15 mg/kg was administered to rats in 6 injections of 2.5 mg/kg each over the course of 3 weeks. Rats received daily prophylactic treatment with Vitamin C via oral gavage one week prior to treatment with DOX, and then for an additional 2 weeks post treatment.[285] In total, the study duration was 6 weeks. A significant decrease in systolic function was observed as a reduction in LVEF in DOX treated rats, as compared to control and Vitamin C treated animals ($p<0.01$).[285] Specifically, 7 of the 9 DOX treated rats presented with a >15% decline in LVEF as compared to control rats ($p<0.01$).[285] Comparatively, all Vitamin C treated animals who received DOX presented with preserved LVEFs that were not significantly different from control animals.[285]

In a separate study by Yu *et al.* (2013), the potential cardioprotective role of the flaxseed constituent ALA was investigated in an acute model of DOX induced cardiotoxicity.[374] In this study, rats were randomly divided into four study groups: i) Saline (n=10); ii) ALA (500 μg/kg; n=10); iii) DOX (2.5 mg/kg; n=10); and iv) ALA+DOX (500 μg/kg; 2.5 mg/kg; n=10).[374] Every animal received 7 injections (every alternate day for 14 days) of the aforementioned study drugs.[374] Echocardiographic analyses demonstrated that the LVEF significantly dropped to 48±4% by the end of the 14-day study in rats treated with DOX (p=0.05). Comparatively, prophylactic administration of ALA was partially cardioprotective with a LVEF of 65±6% (p=0.05).[374]

Finally, in a recent study by Thandavarayan *et al.* (2015), the potential cardioprotective effect of Sch B was tested in an acute male mouse model of DOX induced cardiac dysfunction.[395] Sch B is a plant lignan that is extracted from the Chinese five-flavor berry *Schisandra chinensi*.[84, 337-339, 359] Sch B extract is typically used for its cardioprotective effects including: lipid lowering properties, anti-atherosclerotic effects, and anti-hypertensive properties. It is a potent anti-oxidative agent with oxygen radical scavenging properties similar to those of SDG, that could provide similar protective effects against DOX induced cardiotoxicity.[84, 337-339, 359] In their study, mice received a one-time injection of DOX (20 mg/kg) and varying doses of Sch B for 5 days including; i) 25 mg/kg/daily; ii) 50 mg/kg/daily; and iii) 100 mg/kg/daily.[395] An additional vehicle group was also included in the study design. Echocardiographic analysis of the mice demonstrated an increase in LVEDD and reduced LVFS in the vehicle+DOX treated mice as compared to control ($p<0.01$).[395] However, treatment with Sch B attenuated this increase in LVEDD and decrease in LVFS ($p<0.01$).[395] Unfortunately, the authors of this paper did not mention which dose of Sch B provided this cardioprotective effect. This is a significant limitation of the study as other investigators will

have to determine what dose of Sch B is needed to provide a cardioprotective effect before they can perform further studies to validate its cardioprotective effects against DOX induced cardiac dysfunction.

Collectively, these basic science studies demonstrate the acute cardiotoxic side effects of DOX and TRZ in both male and female animal models.[54, 57, 60, 64, 285, 374, 381, 394, 395] However, these studies primarily focused on acute *in vivo* models of DOX+TRZ mediated cardiotoxicity. [54, 57, 60, 64, 284, 366, 373, 385, 38] Typically, when pre-clinical animal models are considered, many acute *in vivo* studies use higher than normal concentrations of DOX+TRZ, which can hinder translation of basic science findings into the clinical setting. For this reason, we performed a chronic dosing study by Milano *et al.* (2014) to better simulate the clinical situation.[76]

In 2014, Milano *et al.* established a chronic murine model of DOX+TRZ mediated cardiotoxicity to more closely mimic the clinical setting.[76] Specifically, mice received a total of 6 i.p injections of DOX over the course of 2 weeks for a total cumulative dose of DOX of 24 mg/kg.[76] Additionally, a cumulative dose of 10 mg/kg of TRZ was achieved via the same method 1 week after treatment with DOX.[76] In this model, echocardiographic and pressure-volume analysis indicated that DOX induced both LV and RV systolic dysfunction and dilation, which were further exacerbated by subsequent treatment with TRZ.[76] Milano *et al.* (2014) noted that treatment with TRZ alone caused a decrease in LV ErbB2 expression with reversible LV systolic dysfunction, but TRZ did not affect HER2 receptor expression or RV performance.[76]

Similar to this chronic *in vivo* mouse model of chemotherapy induced cardiotoxicity,[76] Akolkar *et al.* (2015) performed a chronic study which investigated the prophylactic use of RAS antagonists for the prevention of DOX+TRZ induced cardiotoxicity.[61] In this study, a total of 240 C57Bl/6 male mice were randomized to one of the following prophylactic treatment regimens: i) Saline (n=60); ii) Aliskiren (50 mg/kg; n=60); iii) Perindopril (3 mg/kg; n=60); or iv) Valsartan (10 mg/kg; n=60).[61] RAS antagonists were administered daily via gavage for 13 weeks.[61] Additionally, mice from each prophylactic treatment arm were further randomized to receive treatment with either: i) TRZ (4 mg/kg; n=20); ii) DOX (4mg/kg; n=20); or iii) DOX+TRZ (n=20).[61] Weekly treatment with the anti-cancer agents was initiated at week 2 and continued for a total of 5 weeks.[61] A cumulative dose of 20 mg/kg of DOX and TRZ was achieved.[61] Serial echocardiographic assessments were performed on awake mice on a weekly basis. At baseline, all echocardiographic indices including HR, PWT, LVEDD, LVFS, and LVEF were within normal physiological limits for all the mice.[61] By week 13, no significant changes in LV dimensions were observed in mice who were treated with TRZ alone.[61] However, mice treated with DOX alone demonstrated a significant increase in LVEDD from 3.2±0.1 mm at baseline to 4.5±0.3 mm at week 13 ($p<0.05$).[61] Similarly, LVEDD significantly increased from 3.1±0.2 mm at baseline to 4.6±0.3 mm at week 13 in mice treated with the combination of DOX+TRZ ($p<0.05$).[61] Interestingly, the prophylactic treatment with either Aliskiren, Perindopril, or Valsartan was able to attenuate this increase in LVEDD in mice administered DOX alone from 4.5±0.2 mm to 3.6±0.2 mm, 3.9±0.2 mm, and 4.0±0.2 mm, respectively at week 13 ($p<0.05$).[61] A similar trend was observed in mice that were treated with the combination of DOX+TRZ where Aliskiren, Perindopril, and Valsartan significantly reduced LVEDD at week 13 from 4.6±0.3 mm to 3.9±0.2 mm, 4.1±0.2 mm, and

4.2±0.1 mm, respectively ($p<0.05$).[61] In this chronic *in vivo* murine model of DOX+TRZ mediated cardiotoxicity, the prophylactic administration of a RAS antagonist was partially cardioprotective.

Our current findings are consistent with the previously validated murine models of DOX+TRZ induced cardiotoxicity.[54, 57, 60, 61, 64, 76, 381, 394] In the current study, in mice that were treated with DOX alone, there was a 27% increase in LVEDD and a 26% decrease in LVEF. However, the prophylactic administration of FLX and its constituents ALA and SDG, were able to attenuate this increase in LVEDD by 50%. Additionally, the consumption of FLX, ALA, and SDG diets minimized the reduction in LVEF in our study to less than 10%, as compared to the RC+Saline group. A similar trend was observed for both echocardiographic indices in the mice that were treated with the combination of DOX+TRZ. To elaborate on these findings, in the mice that were treated with the combination of DOX+TRZ, there was a 32% increase in LVEDD and a 36% decrease in LVEF ($p<0.05$). However, the prophylactic administration of FLX and its constituents ALA and SDG, were able to attenuate this increase in LVEDD by 46%. Additionally, the FLX, ALA, and SDG diets minimized the reduction in LVEF to only 14%, as compare to RC+Saline. Of interest, all three dietary interventions (FLX, ALA, and SDG) provided similar levels of protection. Specifically, the LVEDD values of the different dietary groups differed by only 0.1 mm in both the mice treated with DOX alone or the combination of DOX+TRZ. Comparatively, there was a slightly larger discrepancy between LVEF values in the DOX only treated animals where the prophylactic administration of FLX provided the highest amount of cardioprotection (66±3%) and ALA the least (63±3%). The LVEF values of the DOX+TRZ treated mice showed little variation between the prophylactic treatment with either FLX, ALA, or SDG groups.

Using a chronic *in vivo* female mouse model to mimic the clinical setting, our study is the first to evaluate the cardioprotective effects of FLX against chemotherapy induced cardiotoxicity. Whether FLX and its substituents ALA and SDG are comparable to Probucol, NACA, RAS antagonists, and/or Vitamin C requires additional studies with direct comparisons between these agents. However, our study adds to the paucity of literature that exists on the potential cardioprotective effects of nutraceutical agents in the setting of DOX+TRZ mediated cardiac dysfunction.

To date, there are few clinical studies that have evaluated the use of agents, whether they be pharmacological or nutraceutical, to prophylactically treat chemotherapy induced cardiotoxicity in the breast cancer setting.[78-80, 284, 319] In 2013, the OVERCOME Trial demonstrated that Enalapril (ACE Inhibitor) and Carvedilol (β-blocker) prevented LVSD in patients who received treatment with anthracycline-based chemotherapies.[80] However, the patient population consisted of individuals who were diagnosed with leukemia or malignant hemopathies, not breast cancer.[80] In the PRADA trial by Gulati *et al.* (2016), women with breast cancer were randomized to receive prophylactic treatment with either Candesartan (ARB) or Metoprolol (β-blocker) while undergoing anthracycline based chemotherapy.[79] Their study demonstrated that the prophylactic administration of Candesartan could alleviate the decline in LVEF that is associated with adjuvant breast cancer therapy that ranged from 10 to 61 weeks in duration.[79] However, no cardioprotective effect of Metoprolol was found and whether the beneficial effect of Candesartan can be sustained required a longer follow up period.[79] Notably, only 20% of the patient population in this study were treated with the coadministration of TRZ, which is known to potentiate chemotherapy induced cardiac dysfunction.[79]

The MANTICORE Trial (2017) was the first to conclude that Perindopril (ACE Inhibitor) and Bisoprolol (β-blocker) could protect against cancer related decline in LVEF. However, this study demonstrated that these agents could not prevent TRZ mediated LV remodeling in patients with HER2 positive breast cancer.[78] Recently, the CECCY Trial (2018) presented negative findings that had evaluated Carvedilol (β-blocker) for the prevention of cardiotoxicity under contemporary anthracycline dosage.[284] Specifically, the authors concluded that Carvedilol was unable to demonstrate cardioprotection.[284] Finally, a recent clinical study that was performed by Calvillo-Argüelles *et al.* (2019) indicated that in women with HER2 positive breast cancer that were treated with DOX+TRZ, the concomitant use of statins was associated with a lower risk of cardiotoxicity.[319] This was a retrospective case-control study that was based on electronic chart review of women with HER2 positive breast cancer.[319] These women were treated with a TRZ-based therapy at a single tertiary centre between the years 2002 – 2013.[319] Patients were identified for the study, if they received a pretherapy MUGA scan with an additional ≥2 scans during treatment.[319] Patients were included into the study, if they received treatment with any statin (regardless of clinical indication) before and during their cancer treatment.[319] For every statin-treated patient enrolled in the study, 2 random age and anthracycline exposure status matched patients were included into the control cohort.[319] These patients did not receive statin treatment before or during their cancer treatment.[319] After an 11 month follow up period, a mean difference in final LVEF of -3.4% (CI: -6.6 to -0.3) was observed between the two cohorts (p=0.034).[319] The authors concluded that in women receiving TRZ chemotherapy, the concomitant use of statins was associated with a lower risk of cardiotoxicity.[319] Although protective effects of pharmacological agents have been cited, there remains discrepancies between trials on the beneficial effects of these

therapies.[78-80, 284, 319] Further studies are warranted in order to better elucidate the effectiveness of these agents in the setting of chemotherapy mediated cardiotoxicity.

Despite having already investigated several potential cardioprotective agents, the Canadian health care system still lacks consensus guidelines for the prophylactic administration of cardioprotective therapies in the cancer population.[161, 167, 254, 256] Our study offers a solution to this problem by providing a natural alternative to standard pharmacological therapies. We are the first to demonstrate a cardioprotective effect of the nutraceutical agents FLX, ALA, and SDG in a chronic murine model of DOX+TRZ mediated cardiotoxicity. With no known side effects, FLX has actually been shown to reduce the progression and accelerate the treatment of breast cancers, making it a suitable agent for the prevention of DOX+TRZ mediated cardiac dysfunction.[85, 180, 363-372, 383] Whether flaxseed is cardioprotective in the clinical setting of breast cancer patients treated with DOX and TRZ requires further study.

6.3 Histological Analysis and Cardiac Fibrosis

Doxorubicin induced cardiotoxicity is primarily characterized by a loss of cellular integrity. [296, 396] This finding has been validated in both basic science animal studies as well as clinical studies.[296, 396] In 1994, Siveski-Iliskovic *et al*. performed an *in vivo* study investigating DOX induced cardiotoxicity. In this study, rats were administered a cumulative dose of 15 mg/kg of DOX over a two-week period.[296] Once the rats were sacrificed, heart tissue was collected and prepared for histological analyses.[296] In this study, electron microscopy was used to visualize the histological manifestation of DOX induced cardiomyopathy.[296] Siveski-Iliskovic *et al*. (1994) demonstrated that in mice treated with DOX, there was significant swelling of the mitochondria, vacuolization

of the cytoplasm, formation of lysosomal bodies, and dilatation of the sarcotubular system.[296] Upon further investigation using a higher magnification, distortion of the cristae within the mitochondria was also observed in DOX treated rats. [296]

In order to translate these basic science findings into the clinical context, an invasive myocardial tissue biopsy is required. A study conducted by Bristow *et al.* (1978) investigated the cellular changes associated with DOX treatment.[396] In this study, RV endomyocardial tissue biopsies were collected from 33 adult cancer patients who presented with symptoms of heart failure after receiving treatment with DOX.[396] The authors concluded that DOX administration was associated with a dose-related increase in the degree of myocyte damage including loss of myofibrils, vacuolization of the cytoplasm, and distention of the sarcoplasmic reticulum.[396] Additionally, 93% of patients who received biopsies presented with myocyte damage after receiving an accumulative dose ≥ 240 mg/m^2 of DOX.[396] The conclusions presented by this study are alarming as the current lifetime cumulative dose of DOX is 500 mg/m^2, more than twice the amount used in this study that was able to cause significant myocyte damage. [1, 91, 92, 103, 396]

In addition to DOX focused studies, clinical investigations have also investigated cardiac remodeling in patients who were treated with TRZ. Over a 4-year period, 38 patients with HER2 positive breast cancer were referred to Ewer *et al.* (2005) for suspected TRZ related cardiotoxicity.[186] RV biopsy samples were collected from 9 women who received a second round of TRZ chemotherapy.[186] The study's main findings concluded that none of these patients displayed any adverse cardiac remodeling effects due to their treatment with TRZ.[186] These

findings suggest that anthracycline therapy is the main driving force that leads to adverse cardiac effects, which are then potentiated with concomitant TRZ therapy.

To date, there is a paucity of studies that have evaluated the role of nutraceutical agents in the prevention of DOX and/or TRZ mediated cardiotoxicity from a histological perspective. In 2007, Li *et al.* (2007) conducted a study that investigated the potential cardioprotective effects of the anti-oxidant Sch B against DOX induced cardiotoxicity.[83] Specifically, this study examined whether Sch B could enhance glutathione redox cycling, as its depression is a known contributor in DOX cardiotoxicity.[83] In this study, DOX induced cardiotoxicity was investigated in an acute mouse and rat model.[83] Animals were administered a single injection of DOX (25 mg/kg) with or without pre-treatment with Sch B.[83] Left ventricles from 18 mice were collected and processed for ultrastructural morphologic examination by electron microscopy.[83] Li *et al.* (2007) demonstrated that Sch B significantly preserved the structural components of the LV as compared to control.[83] In a separate study conducted by Xu *et al.* (2011), the cardioprotective role of Sch B was evaluated in a chronic *in vivo* rat model of DOX induced cardiotoxicity.[397] Specifically, rats were administered either a vehicle or Sch B (50 mg/kg) 2 hours prior to treatment with DOX (2.5 mg/kg) for 5 consecutive weeks (cumulative dose of DOX 12.5 mg/kg).[397] Twelve weeks after the last treatment, the rats were sacrificed, and heart tissue was collected for histological and ultrastructural analyses.[397] The authors found that pre-treatment with Sch B significantly attenuated DOX induced damage of the cardiomyocytic structure, which was characterized by disorganization of myofibrillar arrays and cytoplasmic vacuolization.[397]

In a study by Akolkar *et al.* (2017), cardiac structure in hematoxylin and eosin-stained LV sections were normal in both the control and Vitamin C treated animals.[285] Both of these groups demonstrated elongated binucleated cardiomyocytes that were arranged linearly with normal striations.[285] In comparison, DOX treated rats demonstrated several alterations in the structure of cardiomyocytes as well as extensive damage to the cardiomyocytes.[285] These included vacuole formation and disarray, as well as loss of myofibrils and alteration in the shape of the nucleus from an elongated to oval shape.[285] However, in the Vitamin C and DOX treated rats, reduced vacuole formation and a normal registry of myofibrils were observed as compared to rats who were treated with DOX alone.[285]

Subsequent studies performed by our group have collectively confirmed the microscopic evidence of extensive myofibrillar degradation and cellular vacuolization in mice treated with DOX.[54, 57, 60, 64, 285] Furthermore, these effects are potentiated when mice are administered the combination therapy of DOX+TRZ. Specifically, mice in these studies were reported to have significantly more myocardial damage when treated with both DOX+TRZ as compared to those mice receiving DOX monotherapy. [54, 57, 60, 64]

The findings from these previous studies are corroborated by our current findings.[54, 57, 60, 64, 285] In our chronic *in vivo* female mouse model of chemotherapy mediated cardiotoxicity, we confirmed significant levels of histological damage to the cardiomyocytes. Specifically, in the mice treated with DOX alone, there was a significant disruption of the myofibrils and loss of cytoplasmic constituents. The prophylactic administration of FLX or ALA was able to attenuate this cellular damage. However, SDG was not found to be cardioprotective. This could be due to the smaller

sample size of this particular study group or the mechanisms involved in DOX cardiotoxicity. We hypothesized that DOX+TRZ cardiotoxicity is caused by the activation of the inflammatory and OS pathways. Furthermore, we specified that ALA would inhibit the inflammatory pathway while SDG would down-regulate the OS pathway. We suspect that while each individual component may modulate some of the harmful effects associated with DOX cardiotoxicity, a stronger protective effect would be associated with FLX. This is likely due to the fact that cross-talk exists between the inflammatory and OS pathways, and that SDG is only able to inhibit the adverse effects of OS.

In the mice treated with the combination of DOX+TRZ, we observed significant myofibrillar degradation, vacuolization, loss of sarcomere integrity, and sarcoplasmic reticulum dilation. However, the prophylactic administration of FLX, ALA, and SDG was able to attenuate these histological manifestations in mice that were treated with DOX+TRZ. In addition to the prophylactic role of pharmacological agents including Probucol, NACA, and RAS antagonists in preventing DOX+TRZ mediated histological injury,[54, 61, 64] our study is the first to add the nutraceutical FLX to this list of cardioprotective agents. Whether women with breast cancer prophylactically consuming FLX prior to treatment with DOX+TRZ can prevent cardiomyocyte injury at the cellular level requires further study.

It has long been accepted that DOX induced cardiotoxicity is characteristically identified by a loss of cellular integrity. [296, 396] However, the recognition of cardiac fibrosis after treatment with DOX has also received substantial interest over the past few decades. In a study performed by Niu *et al.* (2009), the interruption of Fas/FasL (Fas Ligation) interaction could protect against Dox mediated

cardiotoxicity in mice.[398] Soluble Fas (sFas) is a known competitive inhibitor of FasL and therefore of interest to the authors as a potential cardioprotective agent.[398] WT and sFas transgenic mice were administrated 4 mg/kg of DOX twice a week for a total of 10 injections (cumulative dose 40 mg/kg).[398] At the end of the 9-week study, the mice were sacrificed and the hearts collected for histological analyses. The hearts were sectioned and stained with either hematoxylin and eosin or Masson's trichrome using standard protocols for histomorphometric analyses.[398] Quantitative assessments for myocardial fibrotic area were then performed on five sections in five randomly selected fields per section and expressed as interstitial collagen volume fraction.[398] Extensive interstitial fibrosis was observed in WT mice treated with DOX with an increased collagen volume fraction (2.4 ± 0.16%), which was much less apparent in DOX treated sFas animals (0.52 ± 0.09%, $p < 0.05$).[398] However, the authors did not observe a difference in the collagen volume fraction between saline treated WT and sFas mice (0.48 ± 0.07 vs. 0.46 ± 0.09%, $p > 0.05$).[398] In this study, a substantially larger cumulative dose of DOX (40 mg/kg) was administered to the study animals, as compared to the dosage used in our current study (24 mg/kg). This larger cumulative dose may have contributed to the amount of fibrosis that was detected in the animals by the end of the study period.

Recently Tian *et al.* (2017) performed a study which investigated the preventive effects of DOX induced cardiomyopathy in rats using targeted non-mitogenic acidic fibroblast growth factor (MaFGF) that was mediated by nanoparticles (NP) and ultrasound targeted MB destruction.[399] In this study, rats were injected with DOX (18 mg/kg) twice a week over the course of 6 weeks.[399] After the animals were sacrificed, cardiac collagen deposition was detected using Masson trichrome stain.[399] Red or deep red normal muscle fibers and blue myocardial interstitial collagen

fibers were observed through a microscopic examination of Masson trichrome stained tissues.[399] Significant cardiac remodeling, including increased cardiac fibrosis, was observed in the hearts of the animals who were administered DOX compared to controls ($p<0.05$).[399] In this study, twice the number of DOX injections (6 injections of 3 mg/kg vs. 3 injections of 8 mg/kg) were administered over a longer time period (6 weeks vs. 3 weeks) to the study animals, as compared to the dosage used in our current study. The cumulative dose of DOX achieved in this study was 18 mg/kg.[399] This concentration is below the minimum dosage required to induce cardiotoxicity in a chronic model (24 mg/kg) according to Milano *et al.* (2014).[76] However, it is important to point out that this study used a chronic rat model whereas the Milano *et al.* (2014) study was performed on mice.[76, 399] Therefore, the alterations in the administration of DOX as well as the different animal model may have contributed to the amount of fibrosis that was detected by the end of the 6-week study period. However, further investigations are required in order to confirm these findings.

In a separate study that was conducted by Akolkar *et al.* (2017), the cardioprotective role of Vitamin C against DOX induced cardiotoxicity was evaluated.[285] In this study, Masson's trichrome staining demonstrated a significant increase in fibrosis in DOX treated animals as compared to the saline control and Vitamin C treated animals.[285] Additionally, significantly lower amounts of fibrosis were detected in the animal treated with DOX and Vitamin C, as compared to DOX only treated animals.[285] In this study, there was a 2-week post treatment period after the last DOX injection where the rats continued to receive prophylactic treatment with Vitamin C via oral gavage.[285]

In our current study, we did not observe any significant cardiac fibrosis in the various treatment groups. Typically, a post treatment period is required in order for substantial levels of cardiac fibrosis to develop within the animals.[285] However, our study lacked a post treatment period as the mice were sacrificed within 1 week after their last injection of DOX and TRZ. Additionally, the cumulative dosage of DOX as well as its administration schedule may play a pivotal role in the development in cardiac fibrosis. Therefore, although Masson's trichrome staining did not demonstrate any evidence of cellular enlargement or cardiac fibrosis in our study, further studies focusing on evaluating extracellular remodeling with collagen deposition, matrix metalloproteinases with a longer post treatment period, and variable treatment schedules are warranted to substantiate our findings.

6.4 Hemodynamics

There have been a number of basic science studies that have shown that FLX modulates cardiovascular disease. It is postulated that this is because of FLX's antiatherogenic effects, anti-inflammatory properties, improvement in vascular contractile function as well as its potent antiarrhythmic action during ischemia reperfusion injury. Of specific importance is the study that was performed by Dupasquier *et al.* (2006) which investigated the limits of FLX's anti-atherogenic abilities and its effects on vascular contractile function.[349] Vascular function and atherosclerosis under prolonged hypercholesterolemic conditions were monitored in New Zealand White rabbits for 6 to 16 weeks.[349] Rabbits were randomized to either: i) regular chow (RC); ii) 10% FLX; iii) 0.5% cholesterol (CH); or iv) 0.5% cholesterol and 10%FLX (CF).[349] Cholesterol feeding caused plasma cholesterol levels to increase and atherosclerosis to develop.[349] However, rabbits in the CF group had significantly less atherosclerotic lesions in the aorta and carotid arteries as compared to

CH rabbits.[349] Despite these promising findings, FLX's anti-atherogenic effects were attenuated by the end of the 16-week study.[349] However, FLX supplementation was able to prevent functional impairment in the vasculature as early as week 6.[349] These findings suggest a potential role in endothelial-dependent vaso-relaxation that may have clinical implications.

In addition to FLX, the lignan Sch B which is similar in composition to the FLX-derivative SDG, has been cited as possessing potent homeostatic properties regarding blood pressure regulation. Specifically, Li *et al.* (2007) performed a study on an acute model of DOX induced cardiotoxicity.[83] In this study, anaesthetized rats underwent invasive blood pressure monitoring using a micromanometer-tipped catheter, which was inserted into the right carotid artery and advanced into the LV under pressure control, as previously described.[83, 400] The authors discovered that pre-treatment with Sch B can normalize the maximal left ventricular systolic pressure (LVSP). These findings suggest that Sch B can regulate blood pressure.[83] Similar findings were recorded by Xu *et al.* (2011) who performed invasive blood pressure monitoring in anaesthetized rats.[397] The findings from these studies are limited as they did not specifically control for changes in blood pressure. Specifically, they were investigating the effects of Sch B on cardiac function not vascular relaxation. However, the findings of these studies added to the paucity of literature that investigated the anti-hypertensive effects of FLX (and similar nutraceuticals) in animal models.

Based on these animal studies that evaluated the effects of FLX (and similar compounds) on blood pressure, Rodriguez-Leyva *et al.* (2013) hypothesized that FLX may possess anti-hypertensive properties in patients with peripheral arterial disease (PAD), as blood pressure is a stong predictor of PAD.[346, 401] Rodriguez-Leyva *et al.* (2013) performed the FlaxPAD clinical trial which

investigated the effects of FLX on the systolic and diastolic blood pressures in patients with PAD.[346] A total of 110 patients were enrolled in this prospective, double-blinded, placebo-controlled, randomized clinical trial and consumed a daily total of 30 g of milled FLX for 6 months.[346] The authors observed a 10 mmHg and 7 mmHg decrease in systolic and diastolic blood pressures, respectively.[346] Based on these findings, FLX was characterized as having one of the most potent anti-hypertensive effects ever achieved by a dietary intervention without the need for a pharmacological agent.[346] Following the success of the FlaxPAD trial, Caligiuri *et al.* (2014) performed a subanalysis of these patients to elucidate the anti-hypertensive mechanisms of FLX.[345] It was discovered that FLX was able to inhibit the production of soluable epoxide hydrolase, a pharmacological anti-hypertensive target.[345] Caligiuri *et al.* (2014) concluded that soluable epoxide hydrolase can alter the plasma concentrations of specific oxylipins. Caligiuri *et al.* (2014) credited soluable epoxide hydrolase as being responsible for the anti-hypertensive effects observed in the FlaxPAD population.[345] In an additional study by Caligiuri *et al.* (2016), the effects of FLX on central aortic blood pressure were evaluated in the FlaxPAD population.[344] A significant reduction in central systolic of 10 mmHg and 6 mmHg reduction in diastolic blood pressures were observed in hypertensive patients, as compared to healthy controls.[344] Again, several oxylipins including: 6kPGF$_{1\alpha}$, 11,12-DiHETrE, 16-HETE, 18-HETE, PGE$_2$, and TXB2 were referenced as potential mediators of FLX's anti-hypertensive properties.[344]

In our murine study, we did not demonstrate any changes in serial noninvasive BP. Specifically, the SBP of the mice treated with either DOX alone or the combination of DOX+TRZ, did not change from baseline to the end of the 6-week study. Similarly, the prophylactic treatment with FLX, ALA, or SDG did not significantly alter SBP. In the Dupasquier *et al.* (2006) study, which

investigated the limits of FLX's anti-atherogenic abilities and its effects on vascular contractile function, rabbits were fed the study diets for a total of 16 weeks.[349] As compared to our current study, our mice were administered the FLX, ALA, and SDG diets for only 6 weeks. Therefore, it may be required that animals are fed the FLX-based diets for a longer duration in order to detect a difference in BP.

In the Li *et al.* (2007) and Xu *et al.* (2011) studies, invasive blood pressure measurements were obtained in sedated animals.[83, 397] In comparison, our study used the non-invasive tail cuff method in awake mice. The differences in hemodynamic measurements could be attributed to different methods of acquisition. Additionally, we performed serial BP assessments in our studies whereas the Li *et al.* (2007) and Xu *et al.* (2011) studies obtained a single BP measurement at the end of the study period.[83, 397] Therefore, the authors could not control if these changes existed before the dietary intervention was introduced. Moreover, the Li *et al.* (2007) study was investigating the effects of Sch B not FLX.[83, 397] While Sch B has similar structural and chemical properties to the FLX substituent SDG, they may not possess the same anti-hypertensive properties.[83] Furthermore, we performed a chronic *in vivo* mouse study whereas Li *et al.* (2007) and Xu *et al.* (2011) performed acute *in vivo* studies.[83, 397] Unfortunately, we cannot compare our study to the clinical studies involving the FlaxPAD population as the models used are too different. However, it is clear that further studies investigating the anti-hypertensive effects of FLX in DOX+TRZ mediated cardiotoxicity in the clinical setting of breast cancer are warranted.

6.5 Cardioprotective role of FLX, ALA, and SDG: Mechanistic insights

Over the past two decades, multiple pathways have been studied in both the basic science and clinical settings in order to determine the underlying mechanisms related to chemotherapy induced cardiac dysfunction. However, DOX+TRZ mediated cardiotoxicity is multifactorial. Several pathways been identified in playing a role in chemotherapy related cardiotoxicity and a degree of cross talk between these pathways exists. Therefore, further research is required in order to add to our overall understanding of the mechanisms involved in DOX+TRZ mediated cardiotoxicity. In the current study, we focused on the following three key mechanisms of: i) inflammation; ii) apoptosis and OS; and iii) mitochondrial abnormalities in DOX+TRZ mediated cardiac dysfunction.

Inflammation

In recent years, a large focus has been placed on the inflammatory pathway and its important role in DOX+TRZ mediated cardiotoxicity.[70-72, 402, 403] Specifically, the changes in expression of the inflammatory markers such as tumor necrosis factor-alpha (TNF-α), nuclear factor kappa-B (NF-κB), and interleukin - 1β (IL-1β) have contributed to our understanding of the role that inflammation plays in chemotherapy mediated cardiac dysfunction. Bozkurt *et al.* (1998) investigated the effects of TNF-α in cardiovascular disease.[71] In this study, osmotic infusion pumps were implanted into the peritoneal cavity of rats and infused either a diluent or TNF-α .[71] The TNF-α infusion was titrated to attain a comparable level to that of patients with heart failure (~80 – 100 U/mL) in the clinical setting.[71] The rats were then serially imaged for 15 days using 2D echocardiography to assess for changes in LV structure and function.[71] Additionally, standard

histological techniques were used to detect structural changes in the LV cardiac myocytes. [71] The results indicated that a continuous infusion of TNF-α could lead to a time dependent reduction in LV function, LV dilation, and cardiomyocyte shortening.[71] However, the authors could not specifically predict how pathophysiologically relevant concentrations of TNF-α would affect the heart in chemotherapy related cardiac dysfunction.[71] The authors were only able to conclude that the inflammatory marker TNF-α was elevated in patients with heart failure.[71]

There have been a number of basic science studies that have evaluated the role of TNF-α and other inflammatory markers in the setting of DOX+TRZ mediated cardiotoxicity. Mukherjee *et al.* (2003) investigated the use of garlic as a potential cardioprotective agent in an *in vivo* rat model of chemotherapy induced cardiotoxicity.[72] In this study, rats were administered either: i) freshly prepared garlic homogenate (500 mg/kg); ii) Probucol (120 mg/kg); or iii) double distilled water (vehicle), for 30 days followed by a single dose of DOX (30 mg/kg).[72] Immunohistochemical analyses on cardiac tissue collected from the rats demonstrated that there was no expression of TNF-α in any region of the control animal hearts.[72] Comparatively, intense TNF-α expression was noted in the subendocardial region of the myocardium and in the coronary arteries of the anthracycline treated rats.[72] It was noted that treatment with Probucol caused a reduction in TNF-α expression in the myocardium, endothelial region, and blood vessels.[72] Furthermore, TNF-α expression was significantly decreased in the garlic treated group and was comparable to the Probucol treated group.[72] Therefore, the study concluded that chronic garlic administration reduced DOX induced TNF-α expression and myocyte injury.[72]

In 2012, Abd El-Aziz *et al.* published a study that evaluated the protective effects of Catechin on anthracycline induced cardiotoxicity.[70] Catechin is found in green tea and has been previously reported by Suzuki *et al.* (2007) to improve LV systolic dysfunction, suppress myocardial inflammation and fibrosis, as well as alter cytokine expression in rat autoimmune myocarditis.[402] In the Abd El-Aziz *et al*. (2012) study, the cardioprotective effects of Catechin were attributed to the suppression of specific inflammatory mediators including NF-κB, TNF-α, inducible nitric oxide synthase (iNOS), and adhesion molecules.[70] In their study, a total of 45 rats were randomized to either: i) control; ii) Adriamycin (76 mg/kg); or iii) Adriamycin+Catechin (76 mg/kg; 140 mg/kg Sigma St. Lowis, USA).[70] After 2 weeks of treatment, the rats were sacrificed, and heart tissue was collected for biochemical and molecular analyses.[70] Specifically, RNA was extracted using the Trizol reagent and the manufacture's protocol.[70] Extracted material was used for reverse transcription polymerase chain reaction (RT-PCR) experiments.[70] The expression of NF-κB, TNF-α, and iNOS were significantly increased in the Adriamycin treated group as compared to controls ($p<0.001$).[70] However, this significant increase in the expression of NF-κB and TNF-α was attenuated by the addition of Catechin ($p<0.001$).[70] The study concluded that Catechin was cardioprotective against Adriamycin induced chemotherapy through its potent anti-inflammatory properties.[70]

Recently, Akolkar *et al.* (2017) performed a study that investigated the cardioprotective role of Vitamin C (50 mg/kg) in DOX mediated cardiotoxicity.[285] The authors of this study cited the inflammatory pathway as being partially responsible for the DOX induced cardiac injury observed in the study.[285] A cumulative dose of 15 mg/kg was administered to rats in 6 injections (2.5 mg/kg

each) over the course of 3 weeks.[285] One week before treatment with DOX, Vitamin C was administered prophylactically to the rats via oral gavage and treatment was continued for an additional 2 weeks.[285] The total study duration was 6 weeks, at which time rats were sacrificed and the hearts were collected for biochemical analysis for inflammatory markers.[285] Specifically, Western blot analysis demonstrated a significant increase in the levels of NF-κB in DOX treated rats, as compared to control ($p<0.01$).[285] Comparatively, the increase in NF-κB expression was significantly attenuated by the administration of Vitamin C.[285] In addition to biochemical analyses, pro-inflammatory cytokines including IL-1β and TNF-α were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit in accordance with the manufacture's specifications.[285] Results from this study indicated that both of these inflammatory markers were significantly elevated in the DOX treated rats, as compared to saline control animals ($p<0.01$).[285] However, the prophylactic administration of Vitamin C attenuated this increase in expression of IL-1β and TNF-α ($p<0.01$).[285]

Several other studies have evaluated changes in the expression of these inflammatory cytokines in the setting of chemotherapy induced cardiotoxicity.[404-408] Specifically, Kalantary-Charvadeh *et al.* (2019) recently demonstrated that the anti-inflammatory agent Micheliolide (MCL) was cardioprotective against DOX induced cardiotoxicity.[404] In this acute *in vivo* model, mice were administered a single dose of DOX (15 mg/kg) and daily MCL at varying dosages (12.5, 25, or 50 mg/kg) for 7 days.[404] Several tissue markers of inflammation including IL-1β, TNF-α, and the NF-kB p65 subunit were examined.[404] The authors found that MCL attenuated the levels of these inflammatory markers in cardiac tissue.[404] Collectively, their findings concluded that MCL is

protective against DOX induced cardiotoxicity as it regulates the inflammatory pathway.[404] In a separate study by Shaker *et al.* (2018), Enoxaparin (ENX) was shown to exert anti-inflammatory effects against DOX induced cardiotoxicity.[405] Specifically, the authors randomized 21 adult male rats into: i) control (n=7); ii) DOX (15 mg/kg; n=&); or iii) DOX+ENX (15 mg/kg; 250 IU/kg/daily for 2 weeks).[405] Cardiac inflammatory markers including IL-1β and TNF-α were evaluated at the end of the 2-week study.[405] Although these markers were significantly elevated in the DOX group, their expression was significantly reduced by the administration of ENX.[405] The authors concluded that ENX is cardioprotective against DOX induced cardiac dysfunction as it attenuates the inflammatory pathway.[405]

An additional study by Benzer *et al.* (2018) investigated the cardioprotective effects of Curcumin against DOX induced cardiotoxicity in an acute *in vivo* rat model.[406] The rats in this study were orally administered Curcumin (100 or 200 mg/kg) daily for 1 week, and cardiotoxicity was induced by a single injection of DOX (40 mg/kg) on day 5 of the study.[406] Cardiac inflammatory markers including NF-kB, IL-1β, and TNF-α were elevated in the DOX treated rats.[406] However, the expression of these inflammatory mediators were significantly reduced in the DOX+Curcumin treated group.[406] This study demonstrated that Curcumin has a cardioprotective effect that is associated with its anti-inflammatory properties.[406] A separate study by Abdel-Daim *et al.* (2017) also assessed the changes in inflammatory markers in an acute model of DOX induced cardiotoxicity including NF-kB, IL-1β, and TNF-α.[407] Once again, these mediators were found to be elevated in the animals who were treated with DOX (30 mg/kg).[407] Collectively, these basic science studies demonstrate that inflammatory pathways are profoundly involved, and partially

responsible for the cardiac injury that arises from treatment with DOX+TRZ.[70-72, 285, 402, 404-409] Further chronic *in vivo* studies of DOX+TRZ mediated cardiotoxicity are required in order to characterize the role of inflammation in the development of chemotherapy induced cardiac dysfunction.

In the current study, we evaluated circulating plasma levels of oxylipins as a marker of systemic inflammation. Oxylipins are a family of oxygenated natural products that are derived from PUFAs.[230] They are formed from fatty acids by COX, lipoxygenase (LOX), or cytochrome P450 epoxygenase enzymes, which involve at least one step of dioxygen-dependent oxidation.[230] Oxylipins circulate freely in the bloodstream and often have physiological significance as they change with varying degrees of disease.[230] We analyzed plasma oxylipin levels for potential biomarkers of DOX+TRZ induced inflammation. The concentration of COX-derived oxylipins significantly increased in the RC+DOX+TRZ mice. However, the concentrations of these COX-derived oxylipins including PGA_2, PGE_2, and $PGF_{2\alpha}$ were significantly reduced in the mice receiving daily prophylactic treatment with either FLX or ALA. According to Nayeem *et al.* (2018) who performed a review of the role of oxylipins in CVD, PGs exert harmful pro-inflammatory effects on the body.[231] Furthermore, Chen *et al.* (2018) specifically cite PGE_2 catabolism as possessing harmful biological activity as it is one of the major lipid mediators that is generated during the onset phase of inflammation. [410, 411] Additionally, Kassem *et al.* (2014) demonstrated that increased levels of PGE_2 were elevated during cardiac injury.[412] Although the FLX and ALA diets were able to downregulate the concentration of COX-derived oxylipins, SDG did not demonstrate a similar finding. As to whether SDG has anti-inflammatory actions in the setting of chemotherapy induced cardiotoxicity requires further study.

In addition to these plasma analyses, we measured the levels of protein expression found within cardiac tissue of the pro-inflammatory mediator NF-κB.[70-72, 215, 219] In the RC+DOX+TRZ treated mice, we observed a significant 2.0-fold increase in NF-κB expression as compared to the RC+Saline control group ($p<0.05$). These findings are consistent with that of Akolkar *et al.* (2017) who demonstrated a significant increase in NF-κB protein expression in DOX treated rats as compared to controls ($p<0.01$).[285] However, daily prophylactic treatment with either FLX, ALA, or SDG were able to significantly down-regulate the expression of this inflammatory marker ($p<0.05$). NF-κB is known to up-regulate other downstream pro-inflammatory biomarkers like tumor necrosis factor-alpha (TNF-α) and interleukin 1-beta (IL-1β), which can lead to cardiac fibrosis and heart failure.[70-72, 215, 219] However, neither of these markers were significantly elevated in any of our treatment groups. These findings indicate that dietary FLX and its bioactive components ALA and SDG are able to offer a protective effect in DOX+TRZ mediated cardiotoxicity by attenuating inflammation.

Given our findings, the prophylactic treatment with FLX, ALA, or SDG against DOX or DOX+TRZ treated mice offered a significant protection of cardiac function as indicated by our echocardiographic data. Our inflammatory studies exemplify a similar degree of rescue and protection by the prophylactic use of these diets, both in the DOX only and DOX+TRZ treated mice. However, other inflammatory pathways may still be involved in DOX+TRZ mediated cardiac dysfunction. In addition to NF-κB, there are two other key transcription factors that modulate the inflammatory response.[215-217] These include hypoxia-inducible factors-1 alpha (HIF-1α) and signal transducer and activator of transcription (STAT).[215-217] In a review by Scholz and

Taylor (2013), the role of HIF in the regulation of the immune response and its contribution to inflammation were investigated in an oxygen deprived state.[216] It was concluded that hypoxia is prominently featured in inflamed tissue and that the inhibition of HIF may have potential therapeutic implications.[216] As the heart requires optimal levels of oxygen in order to function properly, HIF expression may be upregulated in cardiac injury that is caused by a DOX activated inflammatory response. However, this has yet to be studied. In a separate review by Kaplan (2013), STAT proteins were cited as playing an intimate role in mediating the responses of target cells to inflammatory cytokines.[217] While cytokines including IL-1β, are integral to the development of inflammation, STAT proteins are crucial to modulating the inflammatory response.[413] Therefore, a further understanding of the involvement of HIF and STAT proteins in the inflammatory pathway is required in order to accurately define their role in DOX+TRZ mediated cardiac dysfunction. Ultimately, cardiotoxicity is likely to be a result of the combined effects of several inflammatory pathways.

Oxidative Stress and Apoptosis

To date, a vast majority of basic science and clinical studies cite programmed cell death or apoptosis as playing a pivotal role in chemotherapy mediated cardiac dysfunction.[54, 64, 207, 295, 414-416] In 1999, Kumar *et al.* investigated the role of Adriamycin induced apoptosis in isolated adult rat cardiomyocytes and whether or not this process could be inhibited by the water soluble anti-oxidant Trolox.[207] In this study, cardiomyocytes were isolated from rat hearts, exposed to Adriamycin (20 μM) for 1 hour, and examined at different post-treatment durations (0-23 hours).[207] Hoechst 33258 staining and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assays were performed on the cardiomyocytes confirming a significant

increase in the number of apoptotic myocytes and nucleosomal fragmentation when exposed to Adriamycin.[207] DNA degradation due to Adriamycin was significantly reduced by Trolox.[207] This study concluded that Adriamycin induced cell death involves both the apoptotic and necrotic pathways, which may be mediated by OS.[207]

Kumar *et al.* (2001) performed a follow up study, which examined the incidence of myocardial apoptosis as well as the changes in expression of apoptotic regulatory gene products in an established animal model of DOX cardiomyopathy.[295] In this chronic *in vivo* model, rats were administered high doses of DOX over a 2-week period for a cumulative dose of 15 mg/kg.[295] Once the rats were sacrificed, the hearts were examined for apoptosis as well as the expression of Bax, Bacl-2, and Caspase 3 at 0, 4, 10, 16, and 21 days after treatment.[295] The degree of apoptosis increased significantly by day 4 but declined at 10- and 16-days post-treatment.[295] However, at day 21 the number of apoptotic cells increased again. Bax expression corresponded to these biphasic changes, whereas the converse was true for Bcl-2 expression which peaked at day 10 post-treatment.[295] The biphasic changes in the incidence of apoptosis were mirrored by the expression of the Bax/Bcl-2 ratio.[295] The expression of Caspase 3 also correlated with increased apoptosis; however, this trend was only observed at early time points.[295] Kumar *et al.* (2001) also investigated the use of Probucol, an anti-oxidant in this study.[295] The authors found that Probucol was able to significantly attenuate the increase in apoptosis as well as the expression of Bax.[295] Overall, the authors concluded that DOX induced apoptosis is mediated by the OS pathway, which may play a role in the development of heart failure.[295]

Similarly, the study by Neilan *et al.* (2007) corroborates these findings in their acute murine model of DOX induced cardiac dysfunction. In this study, WT mice administered a single dose of DOX (20 mg/kg) developed severe cardiac dysfunction.[394] TUNEL assay was used to detect cardiac cell death in mice treated with DOX.[394] Specifically, 24 hours after WT mice received a single injection of DOX, the frequency of TUNEL-positive cardiac nuclei dramatically increased indicating that there was a significantly larger number of cells who were undergoing programmed cell death ($p<0.001$).[394]

Both DOX and TRZ therapy been linked to increased cellular apoptosis. A study conducted by Grazette *et al.* (2004) investigated the effects of erbB2 inhibition by the anti-erbB2 antibody TRZ on cardiomyocyte survival.[116] In this study, primary cultures of neonatal rat ventricular myocytes exposed to TRZ (7.5 mug/ml) for 24 hours were studied for cell viability and apoptosis.[116] The results of this *in vitro* study demonstrated that the administration of TRZ was associated with a significant increase in the expression of the pro-apoptotic Bcl-2 family protein Bcl-X_S and decreased levels of anti-apoptotic Bcl-X_L.[116] The increased ratio between these two proteins is indicative of an increased rate of apoptosis.[116] Additionally, TUNEL staining and propidium iodide flow cytometry that were performed in this study revealed increased rates of apoptosis in the cardiomyocytes that were treated with TRZ.[116]

In 2009, Jassal *et al.* explored the combined effects of DOX+TRZ in an acute murine model of chemotherapy induced cardiotoxicity.[57] In this study, the combination treatment of DOX+TRZ in WT male mice conferred the highest degree of apoptosis at day 10.[57] This chemotherapy-induced apoptosis was characterized by dramatic increases in the pro-apoptotic proteins PARP, Caspase-

3, as well as the aforementioned Bax/Bcl-X_L ratio.[57] This study confirmed that the combination treatment with both anti-cancer drugs potentiates the apoptotic effects as compared to monotherapy with either drug alone.[57] To further corroborate these findings, a study by Walker *et al.* (2011) showed that male WT mice that were treated with the combination of DOX+TRZ developed more pronounced cardiac dysfunction than the mice administered either drug alone.[54] In this study, the Bax/Bcl-X_L ratio significantly increased at day 10 of the acute study.[54] Additionally, this study investigated the cardioprotective role of the anti-oxidant Probucol in DOX+TRZ mediated cardiac dysfunction.[54] The study demonstrated that Probucol was able to improve survival, preserve cardiac systolic function, and attenuate apoptosis.[54]

In a more recent study conducted by Goyal *et al.* (2016), the degree of apoptosis was also measured in an acute *in vivo* murine model of DOX+TRZ induced cardiotoxicity.[64] Specifically, mice were administered a one-time dose of DOX (20 mg/kg) and/or TRZ (10 mg/kg) and then followed for a 10-day period.[64] Once the mice were sacrificed, hearts were collected and prepared for Western Blot analysis of key molecular markers of apoptosis. In mice receiving DOX alone or the combination of DOX+TRZ, the expression of Caspase 3 was increased 3- and 4- fold, respectively ($p<0.05$).[64] The expression of the Bax/Bcl-X_L ratio demonstrated a similar trend in the DOX and DOX+TRZ treated groups increasing by a 1.5- and 1.9- fold change, respectively ($p<0.05$).[64] In 2017, Akolkar *et al.* noticed a significant increase in the expression of the Bax/Bcl-X_L ratio in rats treated with DOX as compared to saline controls or animals who received prophylactic treatment with Vitamin C ($p<0.01$).[285]

Based on these studies, it is suggested that apoptotic pathways are intimately involved, and in part, responsible for the cell death of cardiac myocytes in animals treated with the combination of DOX+TRZ.[54, 57, 61, 64, 116, 394] Further chronic *in vivo* studies of DOX+TRZ mediated cardiotoxicity are needed in order to characterize the role of apoptosis and OS in the development of chemotherapy induced cardiac dysfunction.

In the current study, we observed a significant 1.8-fold increase in the expression of the protein PARP in the RC+DOX+TRZ treated mice, as compared to the RC+Saline control group ($p<0.05$). However, daily prophylactic treatment with FLX, ALA, or SDG was able to prevent this significant increase in PARP expression ($p<0.05$). In addition to PARP, we demonstrated that the levels of Bax protein increased significantly while the amount of anti-apoptotic Bcl-X_L decreased dramatically in mice who were treated with the combination therapy of DOX+TRZ ($p<0.05$). Once again, pre-treatment with FLX, ALA, or SDG was able to significantly attenuate this increase in the Bax/Bcl-X_L ratio ($p<0.05$). Both of these findings suggest that the rate of apoptosis was significantly higher in mice that were treated with DOX+TRZ, as compared to saline controls. Moreover, the prophylactic treatment with FLX, ALA, or SDG was able to prevent this increased rate of apoptosis. These findings indicate that FLX, ALA, and SDG may be able to offer a cardioprotective effect in DOX+TRZ mediated cardiotoxicity by attenuating the degree of apoptosis, similar to previous studies using Probucol, NACA, RAS Antagonists, statins, and Vitamin C. [54, 57, 61, 64, 116, 285, 394]

In addition to tissue analyses, we measured the concentration of circulating oxylipin levels in the mouse plasma. These analyses demonstrated that the levels of the oxylipin 8,9-DiHETrE were

significantly elevated in the RC+DOX+TRZ treated mice. 8,9-DiHETrE is an upstream activator of PPAR-α, which is highly expressed in the heart.[73-75, 237-239, 241-247, 417] PPAR-α is responsible for cardiac fatty acid oxidation which can lead to the accumulation of OS mediators.[73-75, 237-239, 241-247, 417] A study by Rahmatollahi *et al.* (2016) demonstrated that PPAR-α inhibition may have cardioprotective properties as there was a simultaneous increase in DOX mediated OS production and plasma PPAR-α levels.[75] As compared to the RC+DOX+TRZ mice, animals that received prophylactic treatment with FLX, ALA, or SDG showed a significant reduction in the concentration of 8,9-DiHETrE. These tissue and plasma analyses suggest that FLX, ALA, and SDG are capable of decreasing the production of oxygen free radicals by partially inhibiting the OS pathway.

In addition to the apoptotic markers described above, other cell death pathways including autophagy, are involved in DOX+TRZ mediated cardiac dysfunction. Under normal physiological conditions, the process of autophagy, functions by removing damaged cellular organelles via a cell recycling mechanism, which aids in the maintenance of homeostasis.[418] Kobayashi *et al.* (2010) have previously described the involvement of autophagy in mice treated with DOX.[419] This study concluded that the microtubule light chain 3 (LC3-II) is a marker for autophagy and it significantly increased in DOX treated mice.[419] A separate study by Zhang *et al.* also showed that mice treated with DOX had a significant increase in the level of pro-autophagy marker LC3-II at 24 hours post treatment.[420] These studies substantiate the potential involvement of autophagy in the development of DOX induced cardiac dysfunction.[418-420] Further investigation is required to determine the role of autophagy in our chronic *in vivo* mouse model of DOX+TRZ mediated cardiotoxicity.

In addition to autophagy, necrosis is another cell death pathway that is involved in DOX mediated cardiotoxicity. Necrosis is defined as the premature rupture of the cellular plasma membrane with subsequent swelling of cytoplasmic organelles.[418] A study by Lim *et al.* (2004) revealed that trypan blue uptake and creatine kinase (CK) release significantly increased in DOX treated cardiomyocytes.[421] Both trypan blue uptake and CK release are signature markers of necrosis.[421] Lim *et al.* (2004) suggested that calpain activation was responsible for the increase in trypan blue uptake and CK release and that inhibition of calpain activity would attenuate necrosis.[421] Further understanding of the involvement of necrosis in DOX+TRZ mediated cardiac dysfunction is required in order to accurately define the role of this cell death pathway. Ultimately, DOX+TRZ mediated cardiotoxicity is likely to be a product of the combined effects of apoptosis, autophagy, and necrosis.

Mitochondrial dysfunction

A few key basic science studies have begun to attribute mitochondrial dysfunction as being a main contributor to chemotherapy induced cardiac dysfunction. In 2002, Regula *et al.* performed a study that supported Bnip3 as a key regulator of ventricular cardiomyocyte mitochondrial function and cell death in a hypoxic environment.[422] Bnip3 is a member of the BH3-only subfamily that is derived from the Bcl-2 proapoptotic protein family.[423] This BH3 domain is known for its role in regulating cell death as it mediates the heterodimerization process of Bnip3 with both anti- and pro-apoptotic proteins.[423, 424] The structure of Bnip3 is unique as it possesses a C-terminal transmembrane domain.[425, 426] This domain is essential for targeting the mitochondria for apoptosis.[425, 426] In 2002, Regula *et al.* observed elevated levels of Bnip3 protein in a chronic *in*

vivo animal model of heart failure.[422] Furthermore, Bnip3 has been cited as playing a critical role in provoking oxidative injury to the mitochondria following treatment with anthracyclines.[427]

Regula *et al.* (2002) reported a 5.6-fold increase in myocyte death in cells that were subjected to hypoxia, as compared to controls ($p<0.05$).[422] Within these ventricular myocytes and in adult rat hearts, a significant increase in Bnip3 expression was detected ($p<0.05$).[422] Moreover, the increase in the levels of Bnip3 protein were observed in an *in vivo* chronic heart failure rat model.[422] Their study of the subcellular fractions documented that Bnip3 was integrated into the mitochondrial membranes of cardiomyocytes during hypoxia.[422] Furthermore, they witnessed that adenovirus mediated delivery of Bnip3 to myocytes was toxic and resulted in an 8.3-fold increase in ventricular myocyte death ($p<0.05$).[422] This study was the first to provide evidence that Bnip3 is involved and can promote mitochondrial defects as well as cell death in cardiac tissue, which can lead to chronic cardiovascular disease.[422]

In a separate study, Diwan *et al.* (2007) observed that inhibition of ischemic cardiomyocyte apoptosis occurred when Bnip3 was knocked out in a mouse model.[428] Diwan *et al.* (2007) evaluated the effects of ablating Bnip3 on cardiomyocyte death, infarct size, and ventricular remodeling following surgical ischemia/reperfusion (IR) injury in mice.[428] Immediately after IR, there were no significant differences observed between WT mice and Bnip3 knockout ($Bnip3^{-/-}$) mice.[428] However, 3 weeks after IR, $Bnip3^{-/-}$ mice exhibited preserved LV systolic function, diminished LV dilation, and decreased ventricular sphericalization.[428] These findings suggest that the inhibition of Bnip3 function promotes greater cardiac recovery after IR injury.[428]

Once a connection between heart failure and Bnip3 expression was established, the field of Bnip3 mediated mitochondrial injury expanded to encompass other areas of cardiovascular disease. Of interest is the link between Bnip3 expression and chemotherapy induced cardiac dysfunction. Recently in 2014, Dhingra *et al.* described a previously unidentified signaling pathway that couples DOX induced mitochondrial respiratory chain defects and necrotic cell death to the Bnip3 protein.[77] In this study, WT or Bnip3$^{-/-}$ mice received a single injection of saline (control) or DOX (20 mg/kg).[77] Serial murine echocardiography was performed on all mice at baseline and then daily for a total of 10 days.[77] Once the mice were sacrificed, hearts were removed and were processed for ultrastructural (EM), Western blot, and quantitative PCR (qPCR) analyses.[77] In contrast to saline treated mice, cellular defects including vacuolization, disrupted sarcomeres, as well as swollen mitochondria with loss of cristae were observed in the mice receiving DOX.[77] However, Bnip3$^{-/-}$ mice that were treated with DOX displayed relatively normal mitochondrial morphology.[77] Additionally, Bnip3 mRNA and protein expression levels were significantly higher in postnatal ventricular myocytes both *in vivo* and *in vitro* after treatment with DOX ($p<0.05$), consistent with previous findings.[77, 422, 428] The results of this study support the idea that increased Bnip3 function underlies the cardiotoxic effects of DOX.[77]

More recently, Dhingra *et al.* (2017) performed a study that investigated the protective effects of Ellagic Acid (EA) on Bnip3 mediated mitochondrial injury and necrotic cell death in anthracycline treated cardiomyocytes.[427] Specifically, postnatal rat cardiac myocytes were isolated from 1 – 2 day old Sprague-Dawely rats and were subjected to primary culture.[77, 427] The cells were treated with EA at a dose of 1-20 μM and/or DOX at a dose of 10 μM.[77, 427] The authors found that activated Bnip3 targeted the mitochondria, as well as triggered fragmentation, mitophagy, and

necrosis.[427] However, EA was able to impair Bnip3's mitochondrial association and suppress mitochondrial fission, mitophagy, and necrosis.[427] Finally, the authors concluded that EA may provide a therapeutic advantage in reducing oxidative injury and cardiac dysfunction in cancer patients who undergo anthracycline based chemotherapy.[427]

In the Akolkar *et al.* (2017) study, the effects of DOX and Vitamin C on apoptosis and mitochondrial dysfunction were also studied.[285] Specifically, the expression of the pro-apoptotic protein Bnip3, was quantified using Western blot analysis.[285] The authors observed a significant increase in Bnip3 protein levels in animals treated with DOX, as compared to saline control ($p<0.01$).[285] In contrast, Vitamin C provided a protective effect by downregulating the expression of this protein ($p<0.01$).[285] The authors went on to state that alteration of the mitochondrial membrane potential by OS or nitration of various proteins are involved in mitochondrial bioenergetics.[285, 429, 430] This could also be contributing to the mitochondrial dysfunction and activation of the apoptotic pathway that was observed in this study. Additionally, the administration of DOX is known to activate several cell death pathways including apoptosis, necrosis, and autophagy.[91, 431] Therefore, these pathways may also play an important role in regulating mitochondrial health within the myocardium. Future studies are warranted to investigate their potential effects.

In the current study, Western blot analysis of cardiac tissue demonstrated a significant increase in the protein levels of Bnip3, a known marker of mitochondrial dysfunction.[77, 285, 422, 427, 428] Specifically, Bnip3 increased 1.9-fold in the RC+DOX+TRZ treated mice, as compared to saline controls ($p<0.05$). However, daily prophylactic treatment with either FLX, ALA, or SDG was

associated with significant attenuation of this trend in Bnip3 expression ($p<0.05$). These findings suggest that FLX, ALA, and SDG are capable of inhibiting the expression of proteins like Bnip3, which play a pivotal role in mediating mitochondrial dysfunction. Further understanding into the involvement of mitochondrial dysfunction in DOX+TRZ mediated cardiotoxicity is warranted in order to accurately define its role in cell death. It is likely that DOX+TRZ mediated cardiotoxicity is a result of the combined effects of not only mitochondrial dysfunction, but inflammation and OS as well.

6.7 Limitations and Future Directions

There are a number of limitations to our study. First, our study only explored the role of FLX in the prevention of chemotherapy induced cardiac dysfunction in a chronic female murine model of DOX+TRZ mediated cardiac dysfunction. As breast cancer development and its concomitant treatment is not exclusively limited to women, future studies are warranted to test the cardioprotective effects of FLX in both sexes.

Second, our study explored the use of FLX in the prevention of chemotherapy induced cardiotoxicity. However, in current practice, standard pharmacological heart failure therapies are administered to patients only after LV systolic dysfunction associated with DOX+TRZ treatment develops. Therefore, future studies are encouraged to evaluate whether FLX can also be used to promote the recovery of function after the development of DOX+TRZ mediated cardiomyopathy.

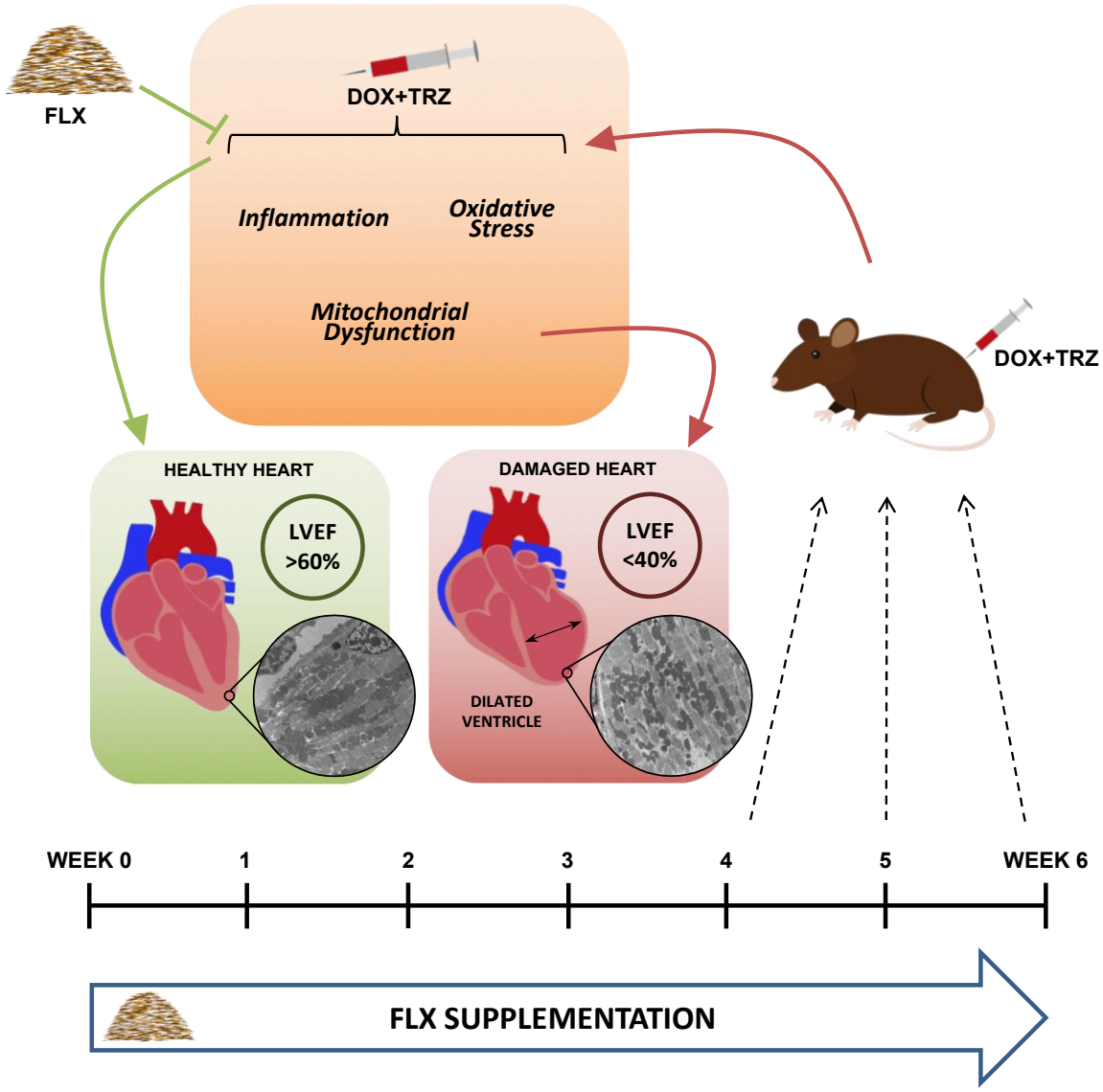
Third, in the current study, we did not evaluate whether FLX, ALA, or SDG are comparable to standard pharmacological therapy that is currently used to treat DOX+TRZ mediated

cardiomyopathy. Therefore, we will perform a comprehensive study in the future to evaluate whether treatment with FLX and/or its components (ALA and SDG) will be incremental to standard pharmacological therapy. Specifically, we will compare whether functional foods such as FLX can provide a comparable and/or incremental improvement in cardiac function as compared to the ACE inhibitor Perindopril, which is a standard pharmacological heart failure therapy that is used to treat cardiac dysfunction that is cause by treatment with DOX+TRZ.

6.8 Clinical Implications

The prophylactic administration of FLX and its substituents ALA and SDG as cardioprotective agents against DOX+TRZ mediated cardiac dysfunction is a novel and clinically applicable finding. Although RAS Antagonists, β-blockers, anti-oxidants, and statins may be useful in the setting of DOX induced cardiotoxicity, their efficacy in DOX+TRZ mediated cardiac dysfunction remains to be explored. Pharmacological agents like Probucol have been proposed as potential cardioprotective agents against DOX+TRZ induced cardiotoxicity, but their clinical use in this setting may be limited due to adverse side effects. FLX is a novel nutraceutical agent with no known side effects; thereby making it a promising cardioprotective agent in DOX+TRZ mediated cardiac damage.

Figure 33: Thesis Summary.



Prophylactic administration of FLX and its constituents partially prevented DOX+TRZ mediated cardiotoxicity by preserving cardiac structure and function. Mechanistically, FLX down-regulated the inflammatory, oxidative stress, and mitochondrial dysfunction pathways and provided a cardioprotective effect. DOX, Doxorubicin; FLX, Flaxseed; LVEF, Left Ventricular Ejection Fraction; TRZ, Trastuzumab.

Chapter 7: Conclusion

Our study demonstrated that the prophylactic administration of FLX, ALA, and SDG partially attenuated DOX+TRZ mediated cardiotoxicity. In our chronic *in vivo* female murine model, FLX, ALA, and SDG: i) significantly improved LV function and cavity dimension; ii) preserved cell structure; iii) did not alter hemodynamics; iv) and diminished the degree of cardiac inflammation, apoptosis, and mitochondrial dysfunction within the myocardium. Future clinical studies are warranted to investigate the role of FLX, ALA, and SDG in preventing the cardiotoxic side effects of DOX+TRZ in the breast cancer setting.

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