Exercise Protection of Vascular Endothelial Cells Against Breast Cancer Chemotherapy Toxicity: Evidence from *In Vitro* Serological Studies

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A thesis submitted in partial fulfilment of the requirements of Edinburgh Napier University for the award of Doctor of Philosophy

September 2021

Abstract of Thesis

5-fluorouracil, epirubicin, cyclophosphamide, and docetaxel is an effective chemotherapy regimen for early-stage breast cancer (BC). However, these drugs associate with a 5% incidence of heart failure (HF). To attenuate the cardiovascular toxicity of chemotherapy, exercise has been proposed as a potential preventative measure. There is now emerging evidence for protective effects of exercise on the heart but there is a lack of evidence for vascular effects, despite vascular endothelial dysfunction being an initiating step in cardiovascular disease (CVD) development.

This study aimed to determine if there are protective effects of habitual physical activity, a single acute exercise session, and an exercise training intervention on chemotherapy-induced vascular endothelial cell toxicity. It was hypothesised that serological factors in active women can alleviate vascular toxicity from chemotherapy; and an acute exercise session and an exercise training intervention can alleviate toxicity in previously sedentary women.

To investigate protective effects of exercise, a novel *ex vivo* method was used. Endothelial cell cultures were preconditioned with serum from active and sedentary woman; woman pre- and post-acute exercise bout; and woman pre- and post-exercise intervention. After 24-hours of serum preconditioning, endothelial cells were exposed to physiological concentrations of 5-fluorouracil, epirubicin, cyclophosphamide, and docetaxel. Cell viability and function, and wound repair were assessed using flow cytometry and scratch assays (to simulate a wound), respectively.

Overall, results confirm that FEC-T chemotherapy drugs, commonly used in early-stage BC treatment, elicit significant damage and dysfunction of endothelial cells. Exercise serum preconditioning from active women, serum collected after an acute exercise session, and serum collected after an exercise training intervention, elicited some protection of endothelial cells against the usual toxicity of 5-fluorouracil, epirubicin, cyclophosphamide, and docetaxel, when compared to control serum preconditioning from inactive women, serum collected prior to an acute exercise session, and serum collected prior to an exercise training intervention, respectively.

Declaration

It is hereby declared that this thesis and the research work upon which it is based were conducted by the author, Marie Mclaughlin.

Marie Mclaughlin

Dedication

I dedicate this thesis to Gary MacDougall (1971-2021). You are the inspiration behind my enthusiasm for exercise oncology. It was an honour to work with you and to become your friend. Thank you.

Acknowledgements

Firstly, I would like to thank the participants who volunteered for the studies presented in this thesis. I must also thank Dr. Katie Hesketh and Dr. Matthew Cocks for their collaboration which made *Chapter 7* possible during COVID-19 restrictions.

I extend my thanks to Russell Wilson, Neil Guthrie, and Marina Mocogni for their technical assistance during data collection in the SES lab; and to Dr. Lesley Young and Sadie Kemp for their technical assistance in 3.C.16.

Thank you to all of the research students for their help and friendship. Especially, Rachael, Dave, Sarah, Filipa, Fern, Bea, Alice, Isla, Connan, and Ying for making 3.C.16 an enjoyable and supportive place to work. Also, thanks to Cameron for allowing me to be the strongest person in the office; and to Dr. Stuart Bailey for my birthday tea bags and protein bars.

To Prof. Geraint Florida-James for sharing his academic expertise and creating thought-provoking discussions which have helped shape me into a critical thinker and the researcher that I am today.

To my Director of Studies, Dr. Mark Ross, who has been nothing short of an exceptional source of support and knowledge throughout the past 3 years. I am truly grateful for the time and effort that you have given to myself and to this project. I could not have done it without you.

Of course, I must thank my mum and dad for their continual support. It is always appreciated.

I would also like to show my appreciation to my friends who have provided much-needed stress relief and laughs throughout my Ph.D.

Finally, a huge thank you to Stephen, for being supportive and understanding, and making my days off enjoyable. I will be forever grateful.

Publications

The following published communications have been produced from the work contained within this thesis. Published communications are provided in **Appendix 21.**

Published Communications

Mclaughlin, M., Florida-James, G., & Ross, M. (2021). Chemotherapy-induced endothelial cell apoptosis and wound repair disruption is attenuated by exercise serum preconditioning. *Medicine & Science in Sports & Exercise*, *53*(8S), 444–445. https://doi.org/10.1249/01.mss.0000764392.41775.1c

Conference Proceedings

Mclaughlin, M., Florida-James, G., & Ross, M. (2021). Chemotherapy-induced endothelial cell apoptosis and wound repair disruption is attenuated by exercise serum preconditioning. American College of Sports Medicine 68th Annual Meeting, 1-5th June 2021.

In vitro HUVEC model of chronic exercise protection of vascular endothelial toxicity of FEC-T chemotherapy treatment for breast cancer using ex vivo serum preconditioning. American Physiological Society – 2020 Integrative Physiology of Exercise Virtual Conference, 5-11th November 2020.

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List of Abbreviations

$\dot{V}O_{2max}$ – Maximum O_2 uptake

5-FU – Fluorouracil

ANOVA – Analyses of variance

ATP – Adenosine triphosphate

ATP – Adenosine-triphosphate

AUC – Area under the curve

BC – breast cancer

BMI – Body mass index

BP- Blood Pressure

Ca_2+ – Calcium

CECs – Circulating endothelial cells

cGMP – Cyclic guanyl monophosphate

CHD – Coronary heart disease

CRF – Cardiorespiratory fitness

CVD – cardiovascular disease

DMSO – Dimethyl Sulfoxide

ECGM – Endothelial Cell Growth Medium

EF HfpEF – Heart Failure with preserved ejection fraction

EMP- Endothelial Microparticles

EPC - Endothelial Progenitor Cell

eNOS – Endothelial nitric oxide synthase

FACS – Fluorescence-activated cell sorting

FdUMP – ***F****luorodeoxyuridine monophosphate*

FdUTP – Fluorodeoxyuridine triphosphate

FEC-T – 5-Fluorouracil, epirubicin, cyclophosphamide, docetaxel

FMD – Flow-mediated dilation

FUTP – Fluorouridine triphosphate

GC – Guanylyl cyclase

GSH – Glutathione

GTP – Guanyl triphosphate

H_2O_2 – Hydrogen peroxide

HCAEC – Human coronary artery endothelial cells

HDL – High density lipoprotein

HF – heart failure

HFrEF – Heart failure with reduced ejection fraction

HR- Heart Rate

HRmax- Maximal Heart Rate

HUVEC – Human umbilical vascular endothelial cells

ICAM – Intercellular adhesion molecule-1

IL- - Interleukin-

IPAQ – International Physical Activity Questionnaire

LDL – Low density lipoprotein

LVEF – left ventricular ejection fraction

mAbs – Monoclonal antibodies

MAPK- Mitogen-Activated Protein Kinase

MEGM – Meso-Endothelial Cell Growth Medium

METs – Metabolic equivalents

MI – Myocardial infarction

NO – Nitric oxide

NT-proBNP – Terminal pro-brain natriuretic peptide

NYHA – New York Heart Association

$O_2 \cdot -$ – Superoxide

PA – Physical activity

PAR-Q – The Physical Activity Readiness Questionnaire

PBS – Phosphate buffer serum

PKG – Protein kinase G

PS – Phosphatidylserine

Chapter 1

Introduction

1.1 Cardiovascular Toxicity of Chemotherapy for Breast Cancer

Breast cancer (BC) is the most common form of cancer, with ~55,000 women diagnosed in the UK in 2017 (World Cancer Research Fund, 2020). BC has an average mortality rate of 40 deaths per 100,000 females (including BC and non-BC females), accounting for 16% of all cancer deaths in women (Smittenaar, Petersen, Stewart, & Moitt, 2016). The BC 10-year survival rate increased over the past 40 years from 40% to 78% in the UK (CRUK, 2020) due to early diagnosis and more effective treatments, including chemotherapy, radiotherapy, surgery, monoclonal antibodies, and target immune therapies (Majeed et al., 2014). In most BC cases treated with chemotherapy, combinations of drugs are used. A common adjuvant chemotherapy regime for women with node-positive early BC is 3 cycles of a combined treatment including 5-fluorouracil (5-FU), epirubicin and cyclophosphamide (FEC), followed by 3 cycles of docetaxel (Roché et al., 2006), as depicted in **Figure 1.1**.

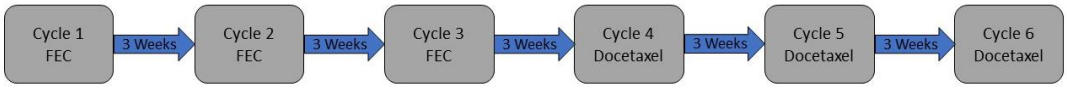

Figure 1.1. FEC-T chemotherapy regimen administered as 3 cycles of FEC followed by 3 cycles of docetaxel, both given every 3 weeks.

This is known as FEC-T treatment and shall be the focus of this research. These drugs are often successful in treating cancerous tumours by damaging tumour DNA and RNA; inhibiting cellular enzymes and tubulin to prevent DNA synthesis; and increasing oxidative stress, effectively inducing tumour cell death (Chighizola, Ong, & Denton, 2011; Gligorov & Lotz, 2004; Longley, Harkin, & Johnston, 2003; Stěrba et al., 2013). However, FEC-T drugs are toxic to the cardiovascular system (Cardinale et al., 2015; Yeh, Courtney, & Bickford, 2009), with cardiovascular disease (CVD) being the leading cause of mortality in BC survivors (Patnaik, Byers, DiGuiseppi, Dabelea, & Denberg, 2011a).

The Cardiac Review and Evaluation Committee (CREC) have stated their criteria for cardiotoxicity of chemotherapy as a reduction in left ventricular ejection fraction (LVEF) of at least 5% to <55% (which signifies a reduced cardiac function) with accompanying signs or symptoms of heart failure (HF), including dyspnoea, fatigue,

oedema, arrhythmias, persistent cough or wheezing, and/or chest pain; or a decline of at least 10% to <55% without HF signs (Seidman et al., 2002). Similarly, a more recent definition of >10 % reduction of LVEF below 53% has been used as a criterion for altering dose or suspending treatment until the normalization of LVEF (Gavila et al., 2017). Three types of cardiotoxicities are currently recognized: acute (occurring during treatment), early-onset chronic (occurring in the first year after treatment), and late-onset chronic (occurring years after treatment) and found to occur with all of the FEC-T treatments (Cardinale et al., 2015; Yeh et al., 2009).

1.2 Mechanisms of FEC-T Toxicity

5-FU exerts a significant deleterious impact on cardiovascular health of BC patients (Yeh et al., 2009)**.** The most common symptom of cardiotoxicity is angina due to myocardial ischaemia, manifesting as ST-segment changes (Cameron, Touyz, & Lang, 2016a). However, arrhythmias, HF, and sudden cardiac death have also been reported (Cameron et al., 2016a; Van Cutsem, Hoff, Blum, Abt, & Osterwalder, 2002). These effects are most likely due to 5-FU metabolites fluorouridine triphosphate (FUTP), fluorodeoxyuridine monophosphate (FdUMP), and fluorodeoxyuridine triphosphate (FdUTP) which cause damage to RNA and DNA; and inhibit DNA synthesis (Longley et al., 2003). This occurs within cells of the cardiovascular system, resulting in activation of the tumour suppressor gene, *p*-53, leading to cellular apoptosis and the detrimental cardiovascular side effects outlined (Longley et al., 2003).

Epirubicin is an anthracycline drug, which account for ~80% of BC treatments (Gavila et al., 2017) and also associates with cardiotoxicity (Daniela Cardinale et al., 2015), and HF (Swain, Whaley and Ewer, 2003). Anthracycline-induced cardiotoxicity is often 'type 1' toxicity which means that the damage to the myocardium is irreversible (Gianni et al., 2008). Anthracyclines work by inhibiting cell division which is effective in obstructive tumour growth. However, this action is non-specific and results in cardiovascular damage, with direct deleterious effects on the heart and vascular endothelium (Ben Aharon et al., 2013; Frias, Lang, Gerber-Wicht, & James, 2010; Kim et al., 2009), subsequently increasing the risk of CVD (Duquaine et al., 2003a).

Cyclophosphamide has also been associated with development of HF which often manifests acutely, depending on dose (Yeh et al., 2009). Additionally, there are reports of increased risk of cardiomyopathy in BC patients given cyclophosphamide, compared to BC patients who did not receiving chemotherapy (Pai & Nahata, 2000). Cyclophosphamide is also associated with vascular complications including hypertension and stroke (Cameron et al., 2016a). Cyclophosphamide has also been associated with development of pulmonary veno-occlusive disease, which is a severe form of pulmonary hypertension, manifesting as progressive obstruction of pulmonary veins (Ranchoux et al., 2015). Cyclophosphamide damages cellular DNA, inducing cell death. This is likely to occur within the vascular endothelium and is a key attributor to increased risk of hypertension and stroke.

Docetaxel is the taxane drug of interest which is usually received in 3 cycles following the 3 cycles of FEC treatment (Roché et al., 2006). Docetaxel is also associated with HF in BC patients (Yeh et al., 2009). Taxanes work by binding and stabilizing tubulin – protein which polymerizes into microtubules to form the cytoskeleton and is required for mitosis. By stabilizing tubulin, taxanes thereby inhibit cell division, leading to apoptosis (Field, Kanakkanthara, & Miller, 2015). Since taxanes work systemically, cellular death occurs in other tissues, including of the cardiovascular system (Peroukides, Alexopoulos, Kalofonos, & Papadaki, 2012), likely responsible for HF development (Yeh et al., 2009).

1.3 Exercise to Counteract Toxicity of FEC-T

Exercise has been proposed as a potential therapeutic to attenuate chemotherapy-induced cardiotoxicity (Kirkham & Davis, 2015). Firstly, the term ‘exercise’ should be defined in order to eliminate ambiguity. Throughout this thesis, the term ‘physical activity’ is in reference to habitual physical activity from observational studies which determine associations; ‘acute exercise’ refers to any single exercise session which has been utilised as an intervention; and an ‘exercise intervention’ is in reference to any chronic exercise intervention comprising of more than one exercise session (usually lasting between 4-16 weeks and comprising of aerobic and/or resistance training several times/week). There is strong epidemiological evidence supporting the benefits of exercise in BC patients, with a meta-analysis of

22 studies showing that there is an inverse relationship between habitual physical activity and all-cause, BC-related death and BC events (Lahart, Metsios, Nevill, & Carmichael, 2015). Furthermore, a meta-analysis found that exercise interventions during chemotherapy treatment significantly reduce systolic blood pressure, reflecting reduced CVD risk (Lewington, Clarke, Qizilbash, Peto, & Collins, 2002; Sturgeon, Ky, Libonati, & Schmitz, 2014b). Another study found that a mixed aerobic and resistance exercise intervention during chemotherapy treatment for BC resulted in decreased Framingham Risk Score, which is a predictor of subsequent 10-year CVD risk (Lee et al., 2019). Therefore, physical activity/exercise has potential to improve cardiovascular health in BC patients and counteract toxicity of chemotherapy treatment.

There are several plausible explanations for reduction of cardiotoxicity risk with exercise. One possible reason is that chronic exercise interventions reduce risk factors for CVD, such as hypertension, hypercholesterolemia, diabetes/poor glucose control, and obesity (Lee et al., 2019a). Additionally, chronic exercise leads to functional adaptations in cardiac structure (Ellison, Waring, Vicinanza, & Torella, 2012), improving cardiac function to allow the heart to provide more oxygen to meet increasing physiological demands at rest and during exercise. Habitual physical activity and chronic exercise interventions also result in improved vascular structure and function with enhanced vasodilatory capacity (Thijssen et al., 2010). This is likely due to increased availability and activation state of key vasodilatory enzyme, endothelial nitric oxide synthase (eNOS), as observed with an exercise intervention in HF patients (Hambrecht et al., 2003). This suggests that physical activity/exercise improves endothelial health which will translate to an improvement in vascular function and overall cardiovascular health, reducing the risk of adverse events.

1.4 The Potential Mechanisms of Exercise Protection

These potential mechanisms draw upon evidence from studies on non-chemotherapy treated populations or from animal studies. The evidence base is scarce with regards to definitive mechanisms of how exercise can help to protect the cardiovascular system from chemotherapy-induced detriments. It is of interest to determine the role of the vascular endothelium as it is the first point of contact

with FEC-T intravenous treatment, and it is of importance in early-stage detection and development of CVD. There is limited mechanistic evidence of the impact of chemotherapy on endothelial cell function and health, and subsequent mechanistic work on exercise to prevent chemo-induced detriments. If more is known about the role of the endothelium in initial toxicity, then it is possible that targeted therapies can be made available for treatment and/or prevention. There are currently only two studies investigating exercise interventions during chemotherapy in BC patients focussing on endothelial function and they have had contrasting findings (Jones et al. 2013; Lee, Kang, et al., 2019). If physical activity/exercise is found to have a protective effect on endothelial cells exposed to chemotherapy, then it can be proposed that exercise can reduce cardiovascular toxicity at an early stage and that exercise should be considered as an adjunct therapy prescribed to BC patients.

The studies comprised within this thesis are aimed at examining the effects of current chemotherapy drugs (FEC-T) on vascular endothelial cell health, and the potential for physical activity, acute exercise, and a chronic exercise intervention to provide protection against any chemotherapy-induced endothelial detriments.

Chapter 2

Literature Review

2.1. Chemotherapy and Cardiovascular Health and Disease

Cardiotoxicity is the leading cause of death in BC patients, attributed to 15.9% of deaths followed closely by BC itself (15.1%) (Patnaik et al., 2011a), highlighting that cardiovascular detriments as a result of the treatment may be more damaging than the cancer itself. Chemotherapy treatment for BC has acute cardiac toxic effects, with 4% of those receiving epirubicin expressing high circulating cardiac troponin (with myocardial damage, this contractile protein degrades and is released into the blood stream) levels (≥0.04ng·ml^{-1}) during treatment, indicating myocardial damage (Mokuyasu, Suzuki, Kawahara, Seto, & Tokuda, 2015). Six cycles of epirubicin-cyclophosphamide chemotherapy produced a significant but sub-clinical increase in cardiac troponin levels from 0.01 ng·ml^{-1} to 0.06 ng·ml^{-1}, occurring alongside reduced LV global area strain (change in the endocardial area from diastole to systole), longitudinal strain (change in myocardial length from diastole to systole), and mitral E/A (ratio of velocity of early filling (E wave) and late diastolic filling (A wave)), despite a non-significant change in LVEF (Chen, Wang, Wu, & Sun, 2019). This indicates that LV diastolic dysfunction may be an early sign of epirubicin cardiotoxicity. Furthermore, abnormal autonomic function (responsible for involuntary regulation of the cardiovascular system) has also been found in BC patients treated with chemotherapy. This results in elevated heart rate and blood pressure at rest, and an exaggerated response to exercise stimuli, and is associated with reduced survivial in cancer patients (Coumbe & Groarke, 2018). In BC patients, 44% of tests of autonomic function conducted in anthracycline-treated patients were abnormal (increase of >26 beats/min and a fall of >20mmHg and >10mmHg in systolic and diastolic pressure, respectively, after 3 minutes of standing (Viniegra et al., 1990)) vs 25% in those receiving CMF (cyclophosphamide, methotrexate, and 5-FU). Only 1 participant in this study showed reduced LVEF, suggesting that cardiotoxicity of treatment can occur without signs of reduced LVEF (Viniegra et al., 1990). This indicates that either LVEF may be an inadequate measure of cardiotoxicity, or clinically relevant toxicity occurs before the manifestation of a reduced LVEF. In contrast, Jensen, Skovsgaard, & Nielsen (2002) found that from pre-treatment to cycle 2, anthracyclines reduced LVEF by ~8% in metastatic BC patients, suggesting an immediate detrimental effect of chemotherapy treatment

on the heart. This difference in findings with LVEF may be due to differing treatment regimens (Jensen et al., 2002).

Cardiac effects progress over time, with longitudinal observational studies showing chronic effects of chemotherapy in BC patients, with symptoms continuing years after treatment. The decreased LVEF has a progressive and delayed response after chemotherapy treatment, with LVEF showing the most marked reduction at 3-3.5 months post-treatment (Cardinale et al., 2015; Jensen et al., 2002), with a continued reduction in LVEF progressing up to 9 months post-treatment (Jensen et al., 2002). Alıcı et al. (2015) found a statistically significant sub-clinical decrease in LVEF from 62.3 to 59.9% at 6-months post-treatment, and Guerra et al. (2016) reported similar findings that cardiotoxicity had an average onset of 6.8 months post-treatment. At 1-year post-treatment, 27% of BC patients developed cardiotoxicity according to the CREC criteria (Guerra et al., 2016), 5% incidence of HF with reduced LVEF, with 70% of HF manifesting as LVEF of 30-45%, and 30% of HF patients experienced HF with LVEF <30% (Cardinale et al., 2002)**.** Overall, findings show that 98% of cardiotoxicity cases occur within the first year of treatment (Cardinale et al., 2015). However, deterioration in systolic function continues to progress at 3-years post-treatment, with 59% of patients exhibiting declines in LVEF by 25% in comparison to their pre-treatment LVEF, with incidence of HF occurring in 20% of cases (Jensen et al., 2002). This cardiotoxicity may never recover fully, with only 11% of patients having full recovery (LVEF increase to baseline levels), and 71% of patients having partial recovery (LVEF increase of <5% and LVEF >50% with no symptoms of HF) at 5 years post-treatment (Cardinale et al., 2015).

Interestingly, impairment in diastolic function appears to be more prevalent than impairment in systolic function, as at 6 months post-treatment, left ventricular diastolic diameter had a significant change of 0.15cm, with no change in LVEF at 6-months post-treatment (Malik et al., 2016). Similarly, at 6 months post-treatment, there was a significant reduction in diastolic function measured as E/A ratio (the ratio of passive and active ventricular filling) from 1.01 to 0.9, indicating clinically impaired cardiac relaxation (Alıcı et al., 2015). At 1-year post-treatment there are similar declines in diastolic function (E/A reduced from 1.3 to 0.86-1.05), with

continued progression of dysfunction at 2 years post-treatment (E/A reduced from 1.3 to 0.89-0.78) (Nagy, Gulacsi-Bardos, Cserep, Hangody, & Forste, 2017a). This progressive toxicity continues to develop at 5 years post-treatment, with 36% of those treated with chemotherapy for BC, showing symptoms of HF (Nagy et al., 2017a). The development of toxicity is denoted in Figure 2.1.

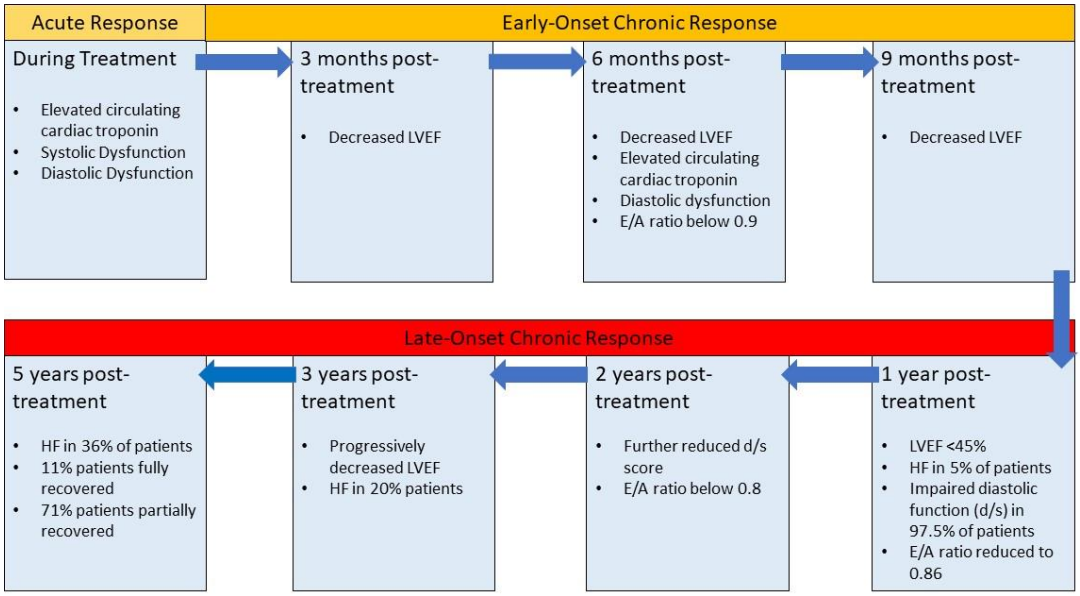

Figure 2.1. Progressive development of cardiovascular toxicity of chemotherapy treatment in breast cancer survivors from during treatment to 5-years post-treatment. LVEF- Left ventricular ejection fraction, HF- Heart Failure.

The damage that chemotherapy inflicts directly upon the heart is predictive upon the outcome of cardiotoxicity for the patient. The level of cardiac damage with chemotherapy as assessed by serum cardiac troponin levels varies between individuals (Adamson et al., 2018) and has a strong correlation with reduced LVEF (*r=-0.93*) (Cardinale et al., 2002). Cardiac troponin levels are predictive of HF development at 12 months, with LVEF progressively decreasing in those with troponin $\geq 0.5 ng \cdot ml^{-1}$ but not in those with troponin $<0.5\ ng \cdot ml^{-1}$, after high-dose chemotherapy treatment (Cardinale et al., 2002). Therefore, toxicity is likely to occur due to direct cardiomyocyte damage, and it can be inferred that those with hearts which are susceptible to damage are most likely to be affected by the cardiotoxic effects of chemotherapy. However, Malik et al. (2016) found a significant change in left ventricular diastolic diameter from pre- to post-chemotherapy, in patients with both elevated and non-elevated cardiac troponin

levels, suggesting that perhaps diastolic dysfunction is more sensitive to chemotherapy exposure than systolic function. The variations in circulating cardiac troponin levels in response to chemotherapy; and the influence of elevated cardiac troponin levels on LVEF and left ventricular diastolic diameter are depicted in **Figure 2.2**.

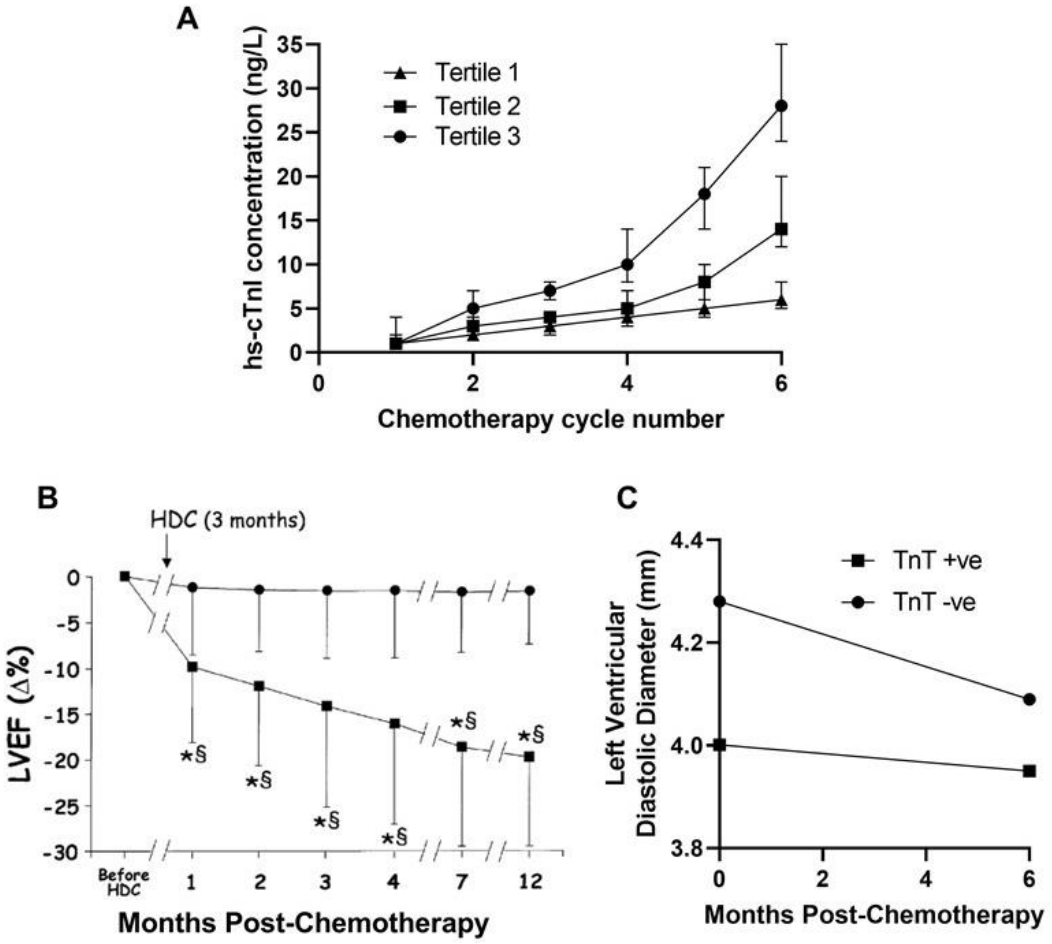

Figure 2.2. Variations in levels of cardiac damage influences changes in systolic but not diastolic toxicity of chemotherapy. (A): High sensitivity troponin concentration (ng/L) at each chemotherapy cycle, displayed as tertiles, adapted from *Adamson et al. (2018)*. (B): LVEF percentage changes during follow-up for those without elevated TnT (circles) and those with elevated TnT (squares), adapted from *Cardinale et al. (2002)*. (C): LVDD (cm) changes during follow-up for those without elevated TnT (circles) and those with elevated TnT (squares), adapted from *Malik et al. (2016)*.

There are several risk factors associated with chemotherapy-induced cardiovascular toxicity, including dose of treatment, combination treatments, pre-existing cardiovascular risk factors, age, and body mass index (BMI). There is a strong association between dose of anthracycline chemotherapy and cardiac toxicity (Wouters, Kremer, Miller, Herman, & Lipshultz, 2005). The dose-dependent toxicity

of anthracycline chemotherapy is illustrated by cumulative doses of 150mg·m^2, 350mg·m^2 and 550mg·m^2 eliciting cardiac event rates of 7, 18 and 65%, respectively (Swain, Whaley, & Ewer, 2003), illustrated in **Figure 2.3**. Furthermore, anthracyclines have a cumulative effect on LVEF, showing reductions from 55% to 50%, 45% and 40% after cycle 3, 4 and 5, respectively, indicating onset of HF with reduced EF (Jensen et al., 2002). This is depicted in **Figure 2.3**. Cardinale et al. (2015) found that cumulative doxorubicin dose has a hazard ratio of 1.09 for cardiotoxicity for each 50mg·m^2 increment, assessed by LVEF. This is similar for cyclophosphamide-based treatments, with HF associated with cyclophosphamide at a prevalence of 7-28%, depending upon dose (Yeh et al., 2009). Similarly, high levels of circulating cardiac troponin were only present in those who received epirubicin doses >400mg·m^2 (Mokuyasu et al., 2015), with concentration increases by 50% with each successive cycle of anthracycline dose (Adamson et al., 2018). Taken together, the data suggests that chemotherapy is cumulatively damaging to the myocardium and that treatment dosage is majorly implicated as a risk factor for toxicity.

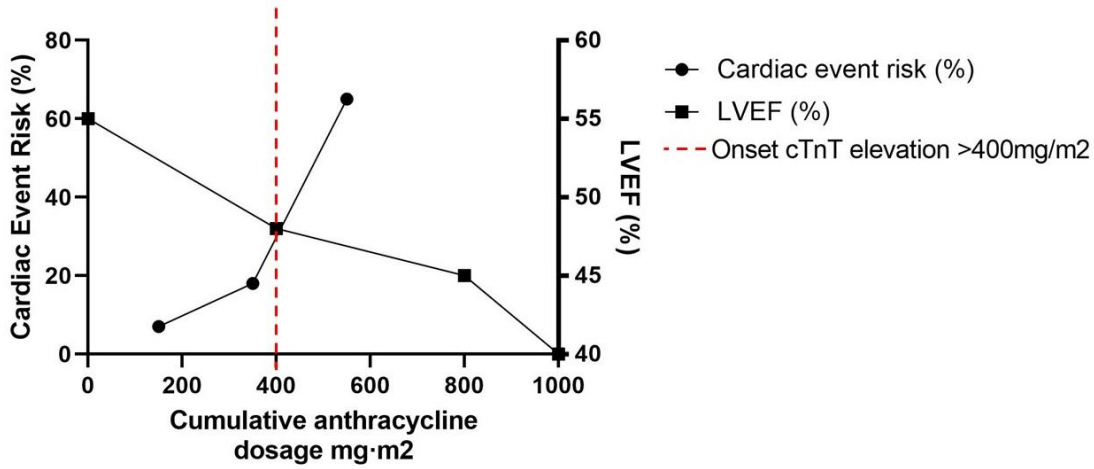

Figure 2.3. Dose response effects of anthracycline drugs on left ventricular ejection fraction (LVEF) and subsequent cardiac event risk (%) with asterisk marking the onset of cardiac troponin T elevation. *Adapted from Swain, Whaley, & Ewer (2003)* and *Jensen et al. (2002)*.

Due to the toxic nature of anthracyclines, taxanes have been used in combination with anthracyclines, with the aim to reduce toxicity. However, a review of 15 RCTs (27,039 patients) found that combination treatment is associated with a statistically

similar risk of toxicity as anthracyclines alone (risk ratio (RR) = 1.56; 95% CI: 0.7-3.05) (Petrelli, Borgonovo, Cabiddu, Lonati, & Barni, 2012). However, when cumulative anthracycline dose is lowered in combination with taxane regimens, lower severe cardiotoxicity (RR = 0.41; 95% CI: 0.26–0.66) and venous thromboembolic events (RR 0.45; 95% CI: 0.26–0.79) were reported. Mortality risk is greater when >3 cycles of anthracyclines precede taxanes in sequential schedules (RR = 2.24; 95% CI: 1.2–4.21) (Petrelli et al., 2012). Therefore, it is likely that anthracycline drugs are causing the most toxicity and therefore, treatment type should also be considered when predicting cardiovascular toxicity.

Moreover, pre-existing CVD and risk factors for CVD are strong predictors of chemotherapy cardiotoxicity, with 38% early-stage and 51% of advanced metastatic BC patients having pre-existing risk factors for cardiotoxicity upon diagnosis (Gavila et al., 2017). Prior to chemotherapy treatment, BC patients had reduced $\dot{V}O_{2peak}$ (the peak volume of O_2 taken up and utilised during intense exercise), and lower LV and RV diastolic function (but preserved LVEF), when compared to age- and sex-matched controls (Beaudry et al., 2019), suggesting a predisposition to cardiovascular dysfunction with BC. Other risk factors such as hypertension, hypercholesterolemia, physical inactivity, smoking, diabetes, and obesity are also strong predictors of cardiotoxicity development with chemotherapy (Barrett-Lee et al., 2009; Gavila et al., 2017; Jones, Haykowsky, Swartz, Douglas, & Mackey, 2007; Yancik et al., 2001). These risk factors are likely to increase susceptibility of the cardiovascular tissues to damage, allowing chemotherapy to further exacerbate the current physiological state. Therefore, pre-existing CVD risk should be considered when determining treatment toxicity risk.

Another risk factor for toxicity is advancing age. Older age is strongly associated with increasing CVD incidence and rates (North & Sinclair, 2012) as well as the risk of chemotherapy-induced cardiotoxicity (Hequet et al., 2004; Swain et al., 2003). Those >65 years are 2.25-fold more likely to develop HF compared to BC patients age ≤65 years receiving the same cumulative anthracycline treatment dose (Swain et al., 2003). The most likely reason behind increased risk of chemotherapy-induced cardiotoxicity with increased age is the worsening of age-related susceptibility to CVD due to prolonged exposure to oxidative stress (Durrant et al., 2009), an

increased predisposition to cell death (Wang et al., 2014), and altered intracellular Ca^{2+} signalling (Soucy et al., 2006). Therefore, patient age is another risk factor increasing the toxic effects of chemotherapy on the cardiovascular system.

BMI has also been found to associate with cardiotoxicity of treatment, as BMI >27 $kg{\cdot}m^2$ significantly associates with LV dysfunction after epirubicin treatment (Fumoleau et al., 2006). LV dysfunction occurred in 0.9% of the BC patients with BMI <27$kg{\cdot}m^2$, compared with 1.8% of BC patients with BMI ≥27$kg{\cdot}m^2$ (Fumoleau et al., 2006). This increased risk of cardiovascular toxicity could be due to overweight/obesity having strong increased risk with CVD in non-cancer populations (Dikaiou et al., 2020). On the other hand, patients with higher BMI receive proportionally larger doses of chemotherapy compared with lower BMI patients, as drugs dosages are calculated as $mg{\cdot}m^2$ and hence, the increased dose may contribute to the increase in cardiotoxicity incidence.

Often, cardiac detriments are preceded by vascular damage and dysfunction (Verma, Buchanan, & Anderson, 2003). In BC patients, acetylcholine-induced changes in forearm blood flow increased 14-days post-chemotherapy, followed by a 36% decrease 1-year post-chemotherapy (Fredslund, Buus, Skjold, et al., 2021). This suggests that early effects of chemotherapy involve enhanced endothelium-dependent vasodilation, with longer-term effects of vascular dysfunction as shown by reduced NO-dependent vasodilation. The increase in vasodilation at 14-days post-chemotherapy occurred alongside increased systemic inflammation, which may have increased NO production, initially increasing vasodilation (Fredslund, Buus, Skjold, et al., 2021). However, long-term systemic inflammation leads to oxidative stress with peroxinitrite production, eNOS uncoupling and NO scavenging, reducing NO bioavailability (Schulz, Gori, & Münzel, 2011). Therefore, this increased systemic inflammation may be the initial step leading to the endothelial dysfunction observed 1 year after completion of chemotherapy, as NO can shift from being protective to harmful in pro-inflammatory states (Deanfield, Halcox, & Rabelink, 2007; Lind et al., 2017).

As vascular dysfunction can lead to cardiac detriments, it is important to assess its clinical implications. In humans, macrovascular endothelial function can be quantified using flow-mediated dilation (FMD). This technique involves measuring

the diameter of an artery before and after a period of occlusion with an inflation cuff. Upon deflation, blood flow is elevated downstream through the artery causing the artery to dilate. This elevation in blood flow, causes shear stress across the endothelium which stimulates eNOS activity, resulting in NO production and release, subsequently causing vasodilation as the smooth muscle is relaxed. Therefore, FMD is an indirect measure of NO bioavailability (Al-Qaisi, Kharbanda, Mittal, & Donald, 2008). FMD is predictive of chemotherapy cardiotoxicity, with every 2.7% increase in FMD associating with a 37% decreased likelihood for LVEF reduction at 3 months post-chemotherapy treatment in BC patients (Anastasiou et al., 2017). Therefore, the vasculature plays an important role in protecting against toxicity, and detriments in endothelial function may be key in the development of cardiac damage.

Furthermore, $\dot{V}O_{2max}$ has been used as a measure of cardiovascular detriments of chemotherapy. However, it must be appreciated that, although the cardiovascular system plays a key role in oxygen uptake, other factors, including muscle metabolic function are also strongly involved (Bassett & Howley, 2000). Therefore, $\dot{V}O_{2max}$ is not a direct measure of cardiovascular function. However, detriments in vascular function result in reduced $\dot{V}O_{2max}$ (Bassett & Howley, 2000). Interestingly, a study found that anthracycline treatment reduced $\dot{V}O_{2max}$, suggesting significant toxicity on the heart, blood vessels and/or skeletal muscle (as these are all contributing factors to $\dot{V}O_{2max}$), despite preservation of LVEF (Brubaker et al., 2019). This suggests that, despite chemotherapy not causing clinical cardiac dysfunction, functional capacity is still impaired.

2.1.2 Mechanisms of Vascular Toxicity of FEC-T Treatments for Breast Cancer

Each individual FEC-T drug has a different mechanism of action and therefore, may have different ways of contributing to tissue toxicity. Until recently, research has focussed on the cardiac mechanisms of toxicity. However, there is now emerging evidence for the negative impact of chemotherapy on the vasculature, and the potential links between these vascular effects of chemotherapy and downstream effects on the cardiovascular system (Ching, Gustafson, Thavendiranathan, & Fish, 2021). Hence, the potential mechanisms of FEC-T toxicity and the pivotal role of the vascular endothelium will now be outlined.

Broadly, FEC-T drugs induce apoptosis within tumours, but this also occurs within vascular endothelial cells. Apoptosis is categorised as programmed cell death (Ching et al., 2021; Erdbruegger, Haubitz, & Woywodt, 2006; Sales et al., 2019; Winther et al., 2016). During apoptosis, the cell is broken down from within by caspase proteins in a 4-step process, involving induction, early-phase, mid-phase, and late-phase (Lawen, 2003). Firstly, chemotherapy-induced damage to DNA/RNA and/or mitochondria, signals activation of *p*-53 tumour suppressor gene to induce the first steps of apoptosis. Activation of *p*-53 is important for tumour suppression as 50% of all cancers have *p*-53 gene mutations (Haupt, Berger, Goldberg, & Haupt, 2003), contributing to evasion of cell death and characteristic tumour cell immortality. The activation of *p*-53 leads to inhibition of flippase (Elmore, 2007), resulting in the inner membrane residue, phosphatidylserine (PS) being translocated from the cytoplasm to the external membrane. This is important as PS facilitates phagocytotic recognition and therefore, the apoptotic cell can be removed from the tissue by circulating macrophages (Lawen, 2003). Interestingly, Annexin V can bind to PS and therefore, this can be used as a marker of apoptosis (Koopman et al., 1994). During the mid-phase of apoptosis, the mitochondria releases Cytochrome C which activates caspase-9 (Elmore, 2007), which is the initial step for caspases activating each other via internal cleavage to separate small and large subunits, in a self-regulating process called the ‘caspase cascade’ (Lawen, 2003). Caspase-9 activates caspase-7 which then activates caspase-3. Caspase-3 is key for the late-phase apoptosis involving DNA degradation and protein degradation via cleavage,

resulting in apoptotic cell shrinking and membrane blebbing. Therefore, cleaved caspase-3 is an ideal marker of late-phase apoptosis. The process of intrinsically induced apoptosis is illustrated in **Figure 2.4**.

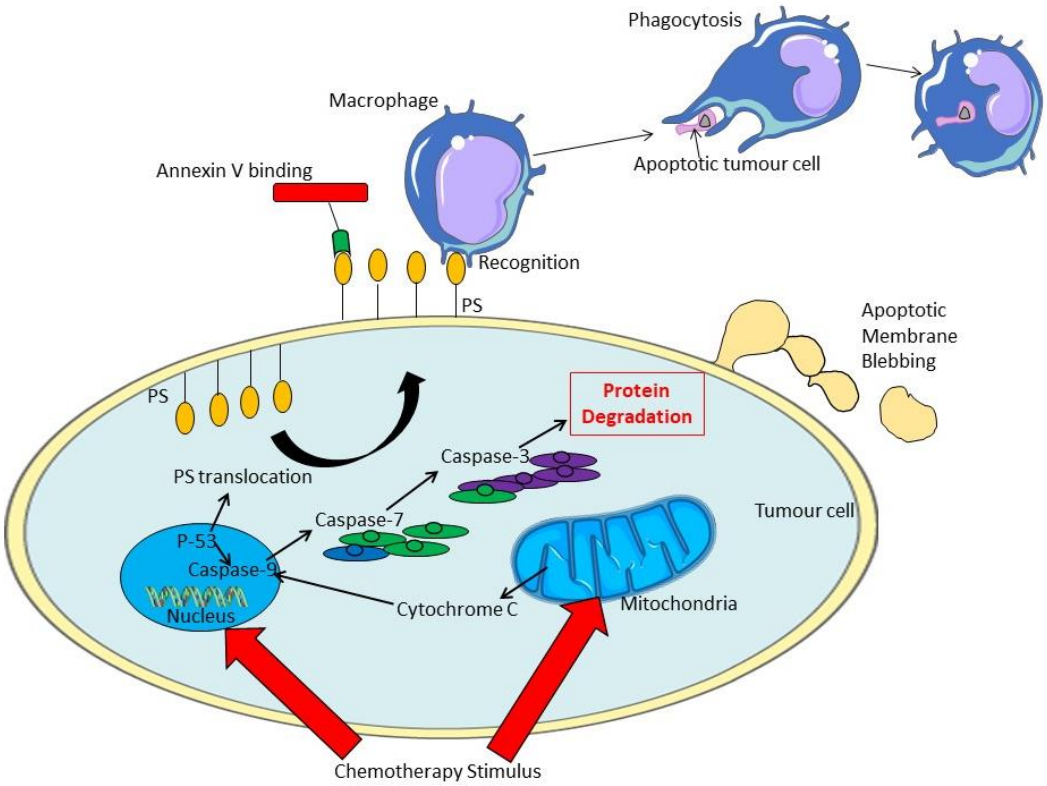

Figure 2.4. Chemotherapy-induced tumour cell apoptosis via *p*-53 activation, including phosphatidylserine translocation to the cell membrane, allowing for detection and identification as an apoptotic cell by phagocytotic macrophage and Annexin V; and activation of the caspase cascade and subsequent protein degradation, resulting in apoptotic blebbing. *Phosphatidylserine (PS).*

2.1.2.1 Fluorouracil

Fluorouracil (5-FU) is an antimetabolite (substance that interferes with the normal metabolic processes within cells, typically by combining with enzymes) which is converted to three main active metabolites within the liver: FUTP, FdUMP, and FdUTP (Longley et al., 2003). These metabolites damage RNA, DNA and inhibit DNA synthesis, resulting in activation of the tumour suppressor gene, *p*-53. This initiates a caspase cascade via several cleavage steps, leading to cellular apoptosis (Longley et al., 2003) – summarised in **Figure 2.5**. Throughout this investigation, 5-FU is investigated as a whole compound as this is in contact with the vasculature before reaching the liver where it is converted to its 3 metabolites. Other studies have also

investigated 5-FU as a whole compound and found toxicity (Kan, Hazama, Maeda, Inoue, Homma, Koido, Okamoto, 2012; Wigmore et al., 2010), and therefore, this is of relevance.

As well as effectively suppressing tumours, 5-FU treatment results in 2% incidence of coronary vasospasm and myocardial ischaemia, with the majority of symptoms developing during the first cycle, commonly manifesting as chest pain (Zafar et al., 2021). The incidence of cardiotoxicity associated with 5-FU varies from 1-68% (depending on dose), with a mortality rate of 2.2-13% (Yeh et al., 2009). Due to turbulent blood flow, the coronary artery is particularly susceptible to endothelial dysfunction and atherosclerosis development because of increased frictional forces causing damage to the endothelial cells (Winther et al., 2016). When combined with exposure to 5-FU, the risk of coronary atherosclerotic development increases, and hence 5-FU associates strongly with myocardial ischaemia, manifesting as ST segment changes, angina, MI, or sudden cardiac death (Cameron et al., 2016a). Coronary artery restriction has been proposed as the most likely underlying mechanism of pathology of 5-FU but only one study has shown evidence supporting this (Kosmas et al., 2008). This may be explained by the direct toxic effects 5-FU exerts on vasculature, causing endothelial senescence and a reduction in eNOS activation (Antonella De Angelis et al., 2017), which is the endothelial specific enzyme responsible for vasodilation (Palmer, Ashton, & Moncada, 1988). As a result of a reduction in eNOS activation/phosphorylation, NO bioavailability is reduced, causing subsequent vasospasm and vasoconstriction (Alter, Herzum, Soufi, Schaefer, & Maisch, 2006; Soultati et al., 2012a), resulting in myocardial ischaemia (Soultati et al., 2012a), systemic hypertension and increased risk of thrombosis (Haddad & Greeno, 2006). Additionally, 5-FU has been found to increase expression of endothelial adhesion molecules, which are involved in the process of atherosclerotic development, giving rise to increased inflammation and further vascular dysfunction (Antonella De Angelis et al., 2017).

2.1.1.2 Epirubicin

Epirubicin is an anthracycline drug which inhibits topoisomerase – the enzyme responsible for unwinding DNA to prevent tangling of the daughter DNA strand to

maintain DNA topology during mitosis. Epirubicin interference of DNA replication prevents mitosis from occurring, resulting in obstruction of tumour growth (Stěrba et al., 2013). Anthracyclines also generate free radicals, causing oxidative stress which results in DNA damage and metabolic dysfunction, leading to activation of caspases which cleave DNA, resulting in apoptosis and tumour cell death growth (Stěrba et al., 2013). These are the mechanisms of action of epirubicin and are effective in preventing tumour growth, but due to the non-specific nature of chemotherapy, is likely to be responsible for cardiotoxicity as the effects also occur within cells of the cardiovascular system. This process is summarised in **Figure 2.5**.

In BC patients, it is reported that the overall incidence of anthracycline-induced cardiotoxicity is 9% (Cardinale et al., 2015), with a 5% incidence of HF (Swain, Whaley and Ewer, 2003), and is often followed by hypertension, tachycardia, and arrhythmias (Wonders, Hydock, Schneider, & Hayward, 2008). Epirubicin induces arterial stiffness and vascular dysfunction. A recent meta-analysis found up to >50% increase in arterial stiffness within 4-6 months of anthracycline treatment (Parr et al., 2020). This is clinically relevant as arterial stiffness is an indicator of vascular health and atherosclerotic development (Boos et al., 2007; Nürnberger, Kribben, Philipp, & Erbel, 2007). Specifically in cancer patients treated with chemotherapy, arterial stiffness has recently been described as a key measure for CVD risk profiling (Parr et al., 2020), with pulse wave velocity (PWV; a non-invasive measure for arterial stiffness) predictive of CVD mortality in BC patients (Parr, Steele, Hammond, Turpin, & Ade, 2021). Anthracycline exposure results in remodelling of the resistance vessels to stiffened blood vessels with reduced compliance (Bosman et al., 2021; Clayton, Brunt, et al., 2021), related to decreased NO-dependent vasodilation (Bosman et al., 2021), and elastin degradation, mediated by TNF-α-dependent vascular inflammation (Clayton et al., 2021).

Moreover, Duquaine et al. (2003) found that brachial artery FMD was reduced from 6.5% to 2.5% in patients after a single dose of anthracycline treatment. Therefore, even acutely, chemotherapy treatment impairs vascular function. These impairments in structural properties and function are likely due to direct and indirect epirubicin-induced endothelial damage. Direct damage to the endothelium causes endothelial cell death which initiates vascular disease via plaque formation

within the site of injury (Werner et al., 2008). Increased cell death also causes a loss of eNOS production (Rippe et al., 2012), leading to vasoconstriction, driving hypertension. Furthermore, endothelial cell death caused by anthracyclines has been found to enhance procoagulant activity, adding more risk for vascular events (Algarni, Greenman, & Madden, 2020). Additionally, indirect anthracycline damage occurs from oxidative stress via production of reactive oxygen species (ROS) (Clayton et al., 2020; Clayton, Hutton, Mahoney, & Seals, 2021; Stěrba et al., 2013). Oxidative stress damages mitochondrial DNA, proteins, and lipids, causing metabolic dysfunction, leading to activation of apoptotic caspases, causing further endothelial cell death (Luu et al., 2018).

Usually, superoxide dismutase (SOD) converts the free radical to hydrogen peroxide (H_2O_2), which is further detoxified by catalase and glutathione peroxidase. However, when ROS production exceeds antioxidant capacity (which is likely with epirubicin exposure), superoxide (O_2·-) radicals scavenge NO, producing a strong vasoconstrictor, peroxinitrite (HNO_3) (Yarana & St. Clair, 2017). Peroxinitrite is responsible for eNOS uncoupling, producing more superoxide molecules, instead of producing NO (Bendall, Douglas, McNeill, Channon, & Crabtree, 2013), resulting in reduced NO bioavailability, with subsequent reduction in vasodilatory capacity, leading to increased blood pressure and arterial stiffness (Luu et al., 2018). This increases strain on the myocardium and likely contributes to HF (Gibbs, Davies, & Lip, 2000). As well as decreasing vasodilatory capacity, a reduction in NO bioavailability increases the likelihood of atherosclerotic plaque formation, likely due to the upregulation of endothelial adhesion molecules (such as intercellular adhesion molecule-1 (ICAM-1)) (Mudau, Genis, Lochner, & Strijdom, 2012a) with chemotherapy treatment (Baeten et al., 2006). This, coupled with increased blood pressure increases the risk of plaque rupture and subsequent cardiovascular events.

Oxidative stress also causes increased permeability of the endothelium, with anthracyclines increasing permeability of bovine arterial endothelial cells by ∼10-fold, resulting in large losses of ATP and the antioxidant glutathione (GSH) from the endothelium (Wolf & Baynes, 2006), further inducing metabolic dysfunction, ROS accumulation, and instigating apoptosis. The enhanced permeability of the endothelium also results in detrimental effects on the myocardium as this creates

an opportunity for passive transportation of anthracyclines into cardiomyocytes, exerting the same cellular effects as observed in endothelial cells, ultimately leading to apoptosis (Ching et al., 2021).

2.1.1.3 Cyclophosphamide

Cyclophosphamide is an alkylating agent. Within the liver, cyclophosphamide is metabolised via P450 to phosphoramide mustard and acrolein (Chighizola et al., 2011). Phosphoramide mustard is the main active metabolite which alkylates nucleophilic basis in tumour DNA, forming crosslinks between and within DNA strands, creating breakages and preventing DNA replication. This damage causes activation of *p*-53 and subsequent caspase cascade and apoptosis of the tumour cell (Chighizola et al., 2011). This process is summarised in **Figure 2.5**. Again, the studies throughout this thesis will focus on the whole compound cyclophosphamide as this is circulating in the bloodstream, and therefore, in contact with the vascular endothelium, before being metabolised within the liver. Cyclophosphamide has been thoroughly utilised in other *in vitro* studies (Kan et al., 2012) and therefore, this was deemed appropriate.

This drug's mechanism of action applies within cells of the cardiovascular system and is associated with vascular complications, including hypertension, MI, and stroke (Cameron et al., 2016a). Cyclophosphamide has also been associated with development of HF which often manifests 1-10 days after the first cycle, with a prevalence of 7-28% in a BC population, depending on dose (Yeh et al., 2009). Vascular toxicity of the drug was assessed in animal models and resulted in a 1% incidence of pulmonary veno-occlusive disease (PVOD), which is a severe form of pulmonary hypertension, resulting in progressive obstruction of pulmonary veins (Ranchoux et al., 2015). Furthermore, treatment with alkylating agents results in a 10% increase in arterial stiffness from baseline to 12 months post-treatment, indicating chronic vascular damage and increased risk of atherosclerotic development (Sekijima et al., 2011).

In an experimental study of combined cyclophosphamide (600 mg·m^2) and doxorubicin (60 mg·m^2) or saline only infusion (administered over 45 minutes), it was found that chemotherapy acutely impaired neurovascular and hemodynamic responses in women with BC, manifesting as increased muscle sympathetic nerve activity and BP. Calf blood flow and calf vascular conductance were decreased during and after chemotherapy infusion, compared with saline only infusion treatment. Vascular damage was also observed through an increase in circulating endothelial microvesicles (Sales et al., 2019). Cyclophosphamide-induced damage to the vasculature can cause neutrophil and monocyte adhesion, followed by platelet accumulation within endothelial lesions (Cameron et al., 2016a). These effects are likely linked to cyclophosphamide-induced reductions in eNOS which results in decreased NO bioavailability (Cameron et al., 2016a; Sekijima et al., 2011), creating a pro-thrombotic environment with activated platelets and upregulated adhesion molecule expression, allowing leukocytes to bind readily to the endothelium (Cameron et al., 2016a; Sekijima et al., 2011). This significantly increases the likelihood of endothelial dysfunction and subsequent development of vascular plaques and cardiovascular events.

Interestingly, it has been proposed that cyclophosphamide damage to the endothelium in the coronary vasculature results in leakage of toxic chemotherapy metabolites, as the usual endothelial barrier is compromised, allowing chemotherapy to directly damage cardiomyocytes (Pai & Nahata, 2000). Therefore, the chemotherapy metabolite is developing an access route for other chemotherapy drugs into the heart, likely causing cardiotoxicity. This damage likely contributes to the cardiomyopathy observed in cyclophosphamide-treated patients as the heart remodels to compensate for areas of cell death (Dhesi et al., 2013). Myocardial ischaemia also occurs with cyclophosphamide exposure with coronary vasospasm is another proposed mechanism of cardiotoxicity (Iqubal et al., 2019; Pai & Nahata, 2000). Together, the effects of cyclophosphamide on the endothelium likely result in cardiotoxicity itself, as well as an increase in the risk of MI in cancer patients. Therefore, it is important to focus research on the vasculature effects of cyclophosphamide as this may be key to the development of cardiac toxicity.

2.1.1.4 Docetaxel

Docetaxel is a taxane, which are drugs identified from genus Taxus plants (yews). The mechanism of action of taxane drugs also involves interference of cancer cell division, mainly through disruption of microtubule function (Gligorov & Lotz, 2004). Microtubules allow the cell to undergo mitosis and regulate intracellular transport, by adapting various formations. Taxanes bind to tumour tubulin, which stabilises the microtubule and preventing depolymerization, inhibiting the process of cell division. This results in the cell cycle stopping at the G_2/M phase in a process of *p-53* dependent cell cycle arrest, subsequently activating apoptosis of the tumour cell. This process is summarised in **Figure 2.5**.

Taxane-induced cardiotoxicity is associated with increased arterial stiffness and oxidative stress (Florescu et al., 2013). There is evidence that docetaxel also increases vascular risk through reducing acetylcholine-induced vasorelaxation, which could be explained by higher vascular-specific H_2O_2 levels than found in BC patients who did not receive chemotherapy (Szczepaniak et al., 2017), similar to that of epirubicin-induced toxicity.

Docetaxel is also associated with a 2.3-8% incidence of HF in BC patients (Yeh et al., 2009), with reductions in LVEF of ~10% from pre- to post-treatment (Florescu et al., 2013). Taxanes such as docetaxel may also directly induce endothelial damage. Evidence for this comes from investigation of the level of circulating endothelial cells (CECs) in the peripheral blood, which are shed from the endothelium in states of inflammation and activation or in response to vascular insult (Erdbruegger et al., 2006) and are negatively associated with endothelial function (Chong et al., 2004). A single dose of taxane chemotherapy treatment increased CEC numbers by >4-fold from pre- to 2-4 hours post-treatment (24 CEC·ml^{-1} to 104 CEC·ml^{-1}), suggestive of substantial endothelial damage after one single treatment (Beerepoot et al., 2004).

Moreover, Florescu et al. (2013) investigated 20 women with BC before and after taxane treatment (cumulative dose of 417±154 g·m^2). After completion of the treatment, patients also exhibited significant elevations in arterial stiffness

(reduced arterial compliance) and oxidative stress. Interestingly, the changes in vascular compliance and oxidative stress are associated with reductions in cardiac function, with LVEF reducing by 7% from pre- to post-treatment (Florescu et al., 2013). This indicates that reductions in cardiac function occur alongside changes in vascular function/structure and therefore, investigating the effects of chemotherapy on the vasculature may help to elucidate the mechanisms behind the physiological process of toxicity on the whole cardiovascular system.

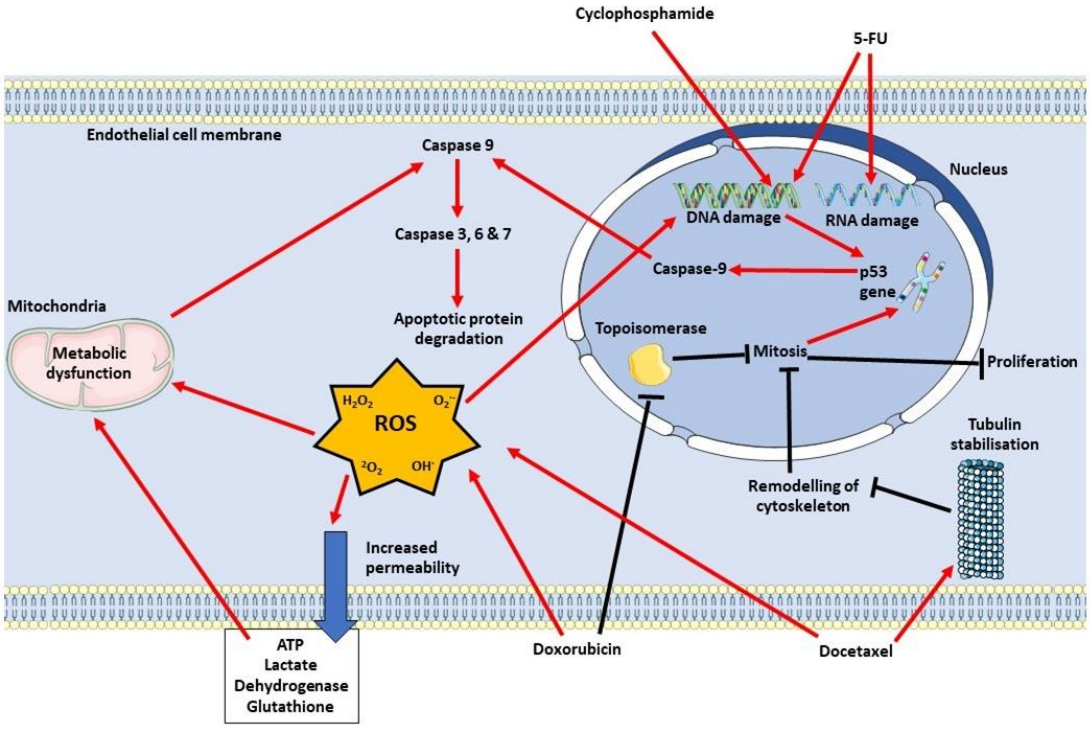

Figure 2.5. Mechanisms of FEC-T-induced endothelial cell apoptosis via activation of caspase cascade. *Reactive oxygen species (ROS), adenosine triphosphate (ATP).*

Together, each FEC-T drug has its own individual mechanism of action, all of which induces death in tumour cells, but also results in deleterious effects on tissues of the cardiovascular system. For a summary of cardiovascular consequences of FEC-T chemotherapy drugs, see **Table 2.1**.

Table 2.1. FEC-T mechanisms of action and adverse cardiovascular effects associated with their administration.

Chemotherapy Drug	Mechanisms of Action	Adverse Cardiovascular Effects
Fluorouracil (antimetabolite)	RNA and DNA damage DNA synthesis inhibition	Hypertension Thrombosis Myocardial ischaemia (S-T segment changes, angina, myocardial infarction) Autonomic dysfunction
Epirubicin (anthracycline)	Inhibits Topoisomerase (Prevents DNA replication) Oxidative Stress (DNA damage and metabolic dysfunction)	Vascular endothelial dysfunction LV systolic dysfunction Heart failure LV diastolic dysfunction Autonomic dysfunction
Cyclophosphamide (Alkylating agent)	DNA alkylation	Autonomic dysfunction Hypertension Myocardial infarction Myocardial ischaemia (Coronary vasospasm) Stroke PVOD Endothelial dysfunction
Docetaxel (Taxane)	Tubulin inhibition (Prevents cell division) Oxidative Stress (DNA damage and metabolic dysfunction)	Arterial stiffness Endothelial dysfunction LV systolic dysfunction

*LV (left ventricular), PVOD (*Pulmonary veno-occulsive disease)

2.2 Exercise and the Protection from Cardiovascular Disease and Potential to Alleviate Chemotherapy Toxicity

As detailed, chemotherapy drugs for BC, either alone, or in combination, result in significant cardiovascular distress, as evidenced by incidence of HF (Cameron et al., 2016a; Swain et al., 2003; Yeh & Bickford, 2009), ischaemia (Cameron et al., 2016a; Iqubal et al., 2019; Pai & Nahata, 2000; Soultati et al., 2012b; Zafar et al., 2021), cardiac remodelling (Pai & Nahata, 2000), significant reductions in endothelial function (Fredslund, Buus, Skjold, et al., 2021), and elevations in cardiomyocyte and endothelial cell apoptosis (Ching et al., 2021; Wolf & Baynes, 2006). This is likely due to drugs having a similar effect on cardiomyocytes as on cancer cells, with inhibition of cardiac enzymes; dysregulation of contractile properties; and increased cell death (Kirwan, McCollum, McDowell, & Byrne, 2015; Stěrba et al., 2013). Combined, these pathophysiological mechanisms increase the risk of cardiovascular events, from thrombosis formation and hypertension, and cardiac dysfunction. Toxicity of chemotherapy on the cardiovascular system is dose-dependent (Adamson et al., 2018; D. Cardinale et al., 2002; Mokuyasu et al., 2015; Swain et al., 2003; Wouters et al., 2005; Yeh & Bickford, 2009), progressive (Cardinale et al., 2002; Malik et al., 2016) and in the case of anthracyclines, irreversible (Gianni et al., 2008), making essential the assessment and management of cardiovascular health during BC treatment. Currently, action to manage and treat toxicity in patient care includes dose limitation and schedule modification (Gavila et al., 2017). However, this is not ideal for the patients as this reduces the efficacy of chemotherapy treatment. Concomitant pharmaceutical interventions have also been used to protect against cardiotoxicity of chemotherapy, including dexrazoxane (Swain et al., 1997), neurohormonal blockers and antihypertensives (Yeh & Bickford, 2009) but these increase treatment costs and may even decrease chemotherapy efficiency (Swain et al., 1997). Added to that, there is paucity of information about longer term effects of using cardioprotective drugs alongside chemotherapy in BC. Therefore, it may be better to implement non-pharmacological interventions to reduce cardiovascular toxicity associated with BC treatment. The following section will detail the pathophysiology of CVD and the protective effects of exercise on the cardiovascular system as a preventative/treatment measure. It is important to understand the mechanisms of

aetiology before understanding how exercise can potentially protect against CVD and chemotherapy-induced cardiovascular toxicity.

2.2.1. Coronary Artery Disease

CAD is a condition of the coronary arteries – the vessels supplying blood to the heart itself, which ensure adequate oxygen supply to the myocardial tissue. In CAD, the coronary arteries are narrowed, usually due to an accumulation of atherosclerotic plaques, instigated by vascular damage and inflammation (Hansson, 2005; Thygesen et al., 2012). This occurs with chemotherapy exposure, particularly well-documented with 5-FU (Cameron et al., 2016a). Narrowing of the vessels results in hypertension, increasing cardiac stress due to pressure overload (Grossman, Jones, & McLaurin, 1975). Restricted coronary arteries also lead to reduction of blood supply and ischaemic areas of the myocardium. If the blood supply becomes entirely blocked, an MI occurs. This is when a sudden deprivation of blood supply to the heart causes apoptosis and necrosis of the cardiomyocytes and alteration of cardiac electrical stimulation, as the myocardium fails to perform systole and diastole (Thygesen et al., 2012). This provides an explanation for chemotherapy-associated myocardial ischaemia, angina, and MI (Cameron et al., 2016a; Kosmas et al., 2008). If the patient survives an acute MI, the myocardium attempts to recover itself via growth of cardiomyocytes. This results in pathological remodelling and hypertrophy, accompanied by fibrous tissue development (Brower et al., 2006), creating mechanical stiffness and cardiac dysfunction, leading to HF (Sutton & Sharpe, 2000), which may explain the occurrence of chemotherapy-induced cardiotoxicity (Yeh et al., 2009). CHF is a complex clinical syndrome resulting from the impaired ability of the heart to cope with the metabolic needs of the body. This is well-documented to occur with FEC-T treatment (Cameron et al., 2016a; Swain et al., 2003; Yeh & Bickford, 2009). A reduction in cardiac function leads to a neurohormonal response, with activation of both the sympathetic nervous system and the renin-angiotensin-aldosterone system (Gibbs et al., 2000). Increased sympathetic activity results in vasoconstriction and increased cardiac output, resulting in increased blood pressure; with the renin-angiotensin-aldosterone system vasoconstriction occurs due to increased bioavailability of endothelin and angiotensin II, and increased expression of vasopressin and

aldosterone leads to sodium and fluid retention, increasing the circulating volume, contributing to hypertension (Gibbs et al., 2000). Together, these regulatory mechanisms induce hypertension, increasing ventricular afterload, increasing myocardial oxygen demand, and decreasing stroke volume. The increased stress on the ventricular wall also stimulates ventricular remodelling, leading to pathological cardiac hypertrophy, which further worsens ventricular function, creating a positive feedback loop for cardiac dysfunction (Gibbs et al., 2000). Reduction in cardiac function through any of the manifestations of CVD reduces the efficiency of blood delivery to vital organs, inducing implications, including dyspnoea, fatigue, congestion, organ damage, and ultimately, cardiovascular mortality (Ikeda, Yamamoto, Yano, & Matsuzaki, 2011). This is outlined in **Figure 2.6.**

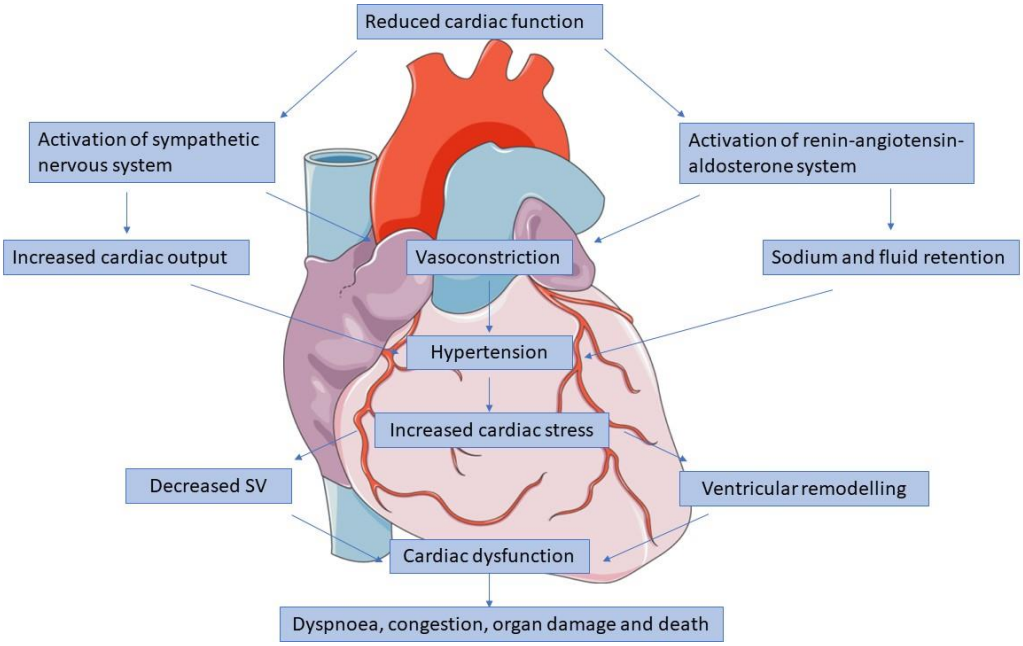

Figure 2.6. Cardiac dysfunction leading to activation of sympathetic nervous system and renin-angiotensin-aldosterone system, resulting in vasoconstriction and hypertension, increasing cardiac stress and dysfunction, resulting in cardiovascular mortality

2.2.2.1 The Importance of the Vascular Endothelium

The vasculature is an efficient network of vessels responsible for the transportation of blood, oxygen, and nutrients to metabolically active tissue, as well as transportation of leukocytes to sites of infection, to name but a few processes. One of the initiating factors of CVD is vascular damage and subsequent vascular

dysfunction. The endothelium is the inner lining of all blood vessels. The endothelium plays a key role in assisting with blood flow regulation, vascular tone, and has anti-inflammatory and anti-thrombotic properties which are essential for prevention of the CVD previously outlined (Verhamme, Hoylaerts, Verhamme, & Hoylaerts, 2016). **Figure 2.7** illustrates the distinct layers of a blood vessel, including the endothelium.

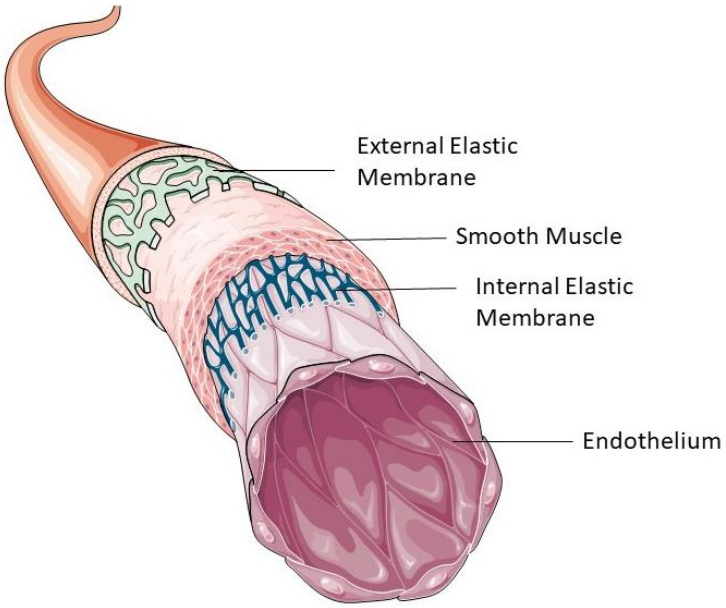

Figure 2.7. Diagram of a blood vessel wall detailing the distinct layers, including the endothelium, internal elastic membrane, vascular smooth muscle, and external elastic membrane.

The endothelium plays a crucial role in vasodilation, which is the mechanism whereby blood vessels increase in diameter to allow increased blood flow to pass through the vessel. This is the main process by which the vasculature controls blood flow distribution. The endothelium plays a central role in this process by producing and releasing nitric oxide (NO; via the enzyme eNOS), which goes on to stimulate vascular smooth muscle relaxation, thus resulting in dilation of the artery. The production and release of NO arises from a complex series of biochemical reactions within the endothelial cell cytosol. Shear stress and acetylcholine act upon the muscarinic receptor to stimulate release of $Ca^{2}+$ from the sarcoplasmic reticulum (SR). The increased $Ca^{2}+$ concentration within the endothelial cell, allows binding of $Ca^{2}+$ to calmodulin to create calcium calmodulin. This complex activates eNOS by phosphorylating the Ser1177 protein. Upon activation, eNOS assists in the

conversion of L-arginine to L-citrulline, resulting in production of NO. NO diffuses across the endothelial layer into the vascular smooth muscle cells. Here, NO activates guanylyl cyclase (GC) which converts guanyl triphosphate (GTP) to cyclic guanyl monophosphate (cGMP), which acts on protein kinase G (PKG), resulting in phosphorylation of the myosin light chain on the myofilaments, causing myosin heads to detach from the actin filament, resulting in relaxation of the smooth muscle cells, and vasodilation of the vessel (Wedgwood, Schumacker, & Steinhorn, 2012). The process of endothelium-dependent vasodilation is illustrated in **Figure 2.8**.

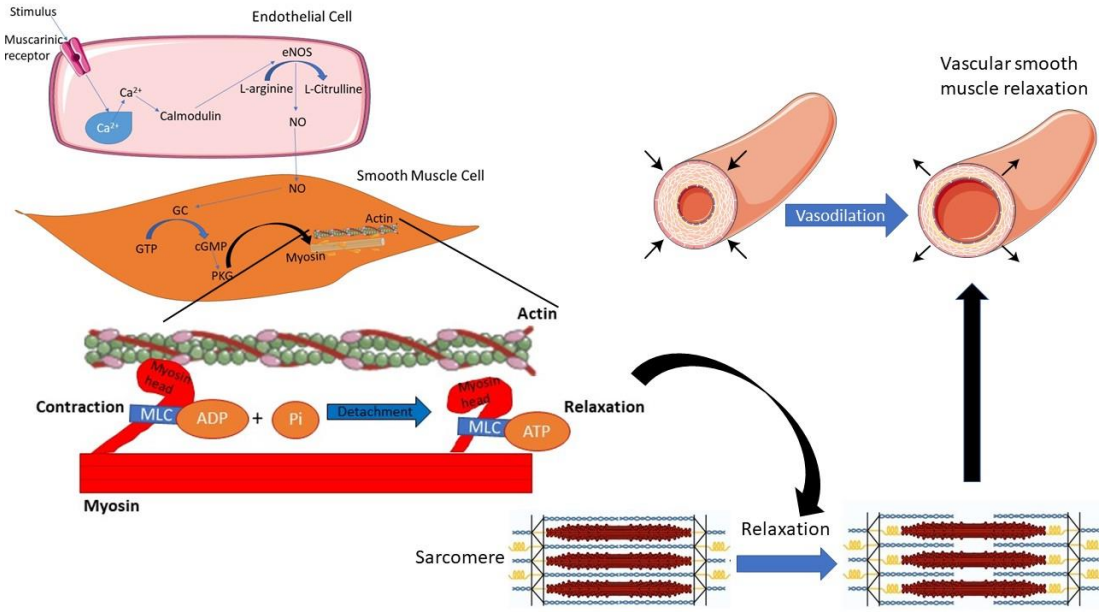

Figure 2.8. Endothelial-dependent vasodilation controlled by the activation of eNOS via phosphorylation of Serine-1177. *SR (sarcoplasmic reticulum), eNOS (endothelial nitric oxide synthase), Nitric oxide (NO), Guanylyl cyclase (GC), guanyl triphosphate (GTP), cyclic guanyl monophosphate (cGMP), protein kinase G (PKG), MLC (myosin light chain).*

This mechanism allows greater blood flow through the vascular bed and therefore, is a key mechanism in controlling blood flow distribution. Additionally, the ability of the vascular endothelium to modulate vessel diameter regulates blood pressure, with vasodilation reducing vascular resistance, and vice versa for vasoconstriction. Hypertension (defined as systolic blood pressure >140mmHg and diastolic blood pressure >90mmHg) leads to the development of CVD, with The Framingham Study reporting hypertension to associate with a two- and three-fold relative risk for HF

and stroke, respectively, compared with normotension (Lloyd-Jones et al., 2002; Wolf, Abbott, & Kannel, 1991). In fact CHD mortality risk doubles for each 20mmHg increase in SBP and each 10mmHg increase in DBP (Lewington et al., 2002). Therefore, efficient blood pressure regulation is essential for reduction of CVD risk. Since the endothelium contributes highly to vasodilatory capacity via NO-mediated mechanisms, maintenance of endothelial health is critical for the maintenance of cardiovascular health (Beaudry et al., 2018).

In addition to blood pressure regulation, endothelial cells maintain vascular haemostasis through its anticoagulant properties, including heparin-like molecules and thrombomodulin; and antiplatelet properties, such as NO and prostacyclin. When the vasculature is subject to injury, these properties are responsible for restoration of vascular integrity by limiting clot formation to the local area of injury and preventing unfavourable adhesion of molecules to other areas of the endothelium (Rajendran et al., 2013). Vascular integrity is also maintained through production of NO, which is anti-atherogenic and plays an important role in reducing atherosclerosis risk (Mudau et al., 2012a). In hypercholesterolemic rabbits, reduction of NO bioavailability by NO inhibitor L-NAME accelerates aortic plaque formation and dysfunction of aortic relaxation in response to acetylcholine stimulation by 24% and 38%, respectively (Naruse et al., 1994). This is paralleled in human studies investigating the relationship between NO bioavailability and atherosclerosis (Oemar et al., 1998). The rate of release and peak concentration of NO after stimulation with calcium ionophore was reduced by 80% and 89%, respectively, in atherosclerotic carotid arteries compared to normal mammary arteries, suggesting NO is protective against atherosclerosis (Oemar et al., 1998). The mechanism behind this is likely NO-mediated inhibition of vascular platelet and leukocyte adhesion and transmigration (Cooke & Tsao, 2011).

Overall, the endothelium is important in maintaining vascular health, by reducing the risk of vascular injury by controlling vascular tone, eliciting anti-coagulant and anti-adhesion properties, and the ability to induce vascular repair, as vascular insult without efficient repair is one of the mediating mechanisms behind the initiation of vascular disease and CVD mortality.

Vascular endothelial dysfunction is a term used to describe abnormal vascular conditions which result from an imbalance between vasodilatory and contracting factors, including an overall reduction in NO bioavailability, an imbalance between procoagulant and anticoagulant factors or between growth-inhibiting and -promoting factors (De Meyer & Herman, 1997). When the normal regulatory processes of the endothelium are off balance, this results in physiological consequence including dysregulated blood pressure, pro-thrombotic and pro-atherogenic phenotype factors (De Meyer & Herman, 1997). Vascular endothelial dysfunction is an initiating step in cardiovascular complications and is implicated in many cardiovascular and metabolic conditions, including hypertension and diabetes (De Meyer & Herman, 1997).

One of the main mediators of vascular endothelial dysfunction is endothelial cell apoptosis (Wang et al., 2014) which increases leukocyte infiltration into the endothelium, leading to endothelial dysfunction (Wang et al., 2014). Endothelial apoptosis occurs in response to apoptotic stimuli, such as chemotherapy (Ching et al., 2021; Erdbruegger et al., 2006; Sales et al., 2019; Winther et al., 2016). During apoptosis, the cell is broken down from within by caspase proteins which is important for removal of damaged cells to prevent unwanted tissue growth, such as cancerous tumours (Lawen, 2003). The process of apoptosis is important for removal of damaged cells (Lawen, 2003) to ensure the endothelium maintains its integrity (Affara et al., 2007). However, in pathological circumstances, such as the endothelium under chemotherapy exposure, upregulation of apoptosis can lead to undesirable removal of cells, damaging the integrity of the endothelial layer. The process of endothelial apoptosis is illustrated in **Figure 2.9**.

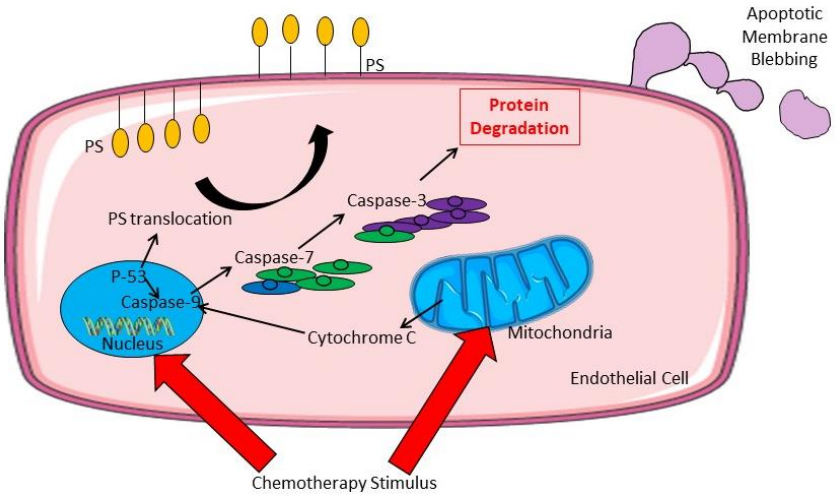

Figure 2.9. Process of chemotherapy-induced endothelial cell apoptosis, including phosphatidylserine translocation from the intracellular to the extracellular membrane; and activation of the caspase cascade and subsequent protein degradation, resulting in apoptotic blebbing. *Phosphatidylserine (PS).*

2.2.2.2 Endothelial Dysfunction and Atherosclerosis Development

Under pathophysiological circumstances, whereby the endothelium is exposed to damage or insult, such as hypertension, smoking, hyperglycaemia, or hypercholesterolaemia, the usual protective regulatory mechanisms of the vasculature, including NO production, are downregulated (Verma et al., 2003). This increases the likelihood of atherosclerosis development, leading to MI or strokes. These account for a high proportion of mortality, with 1 in 5 and 1 in 18 deaths in the US caused by CHD and stroke, respectively (Lloyd-Jones et al., 2009). Atherosclerosis development is a complex and multi-factorial phenomenon, involving inflammatory-mediated pathology. Usually, low density lipoproteins (LDLs) present in the blood pass through the endothelial cell layer by transcytosis and are then oxidised in the tunica media (Sullivan, Sarembock, & Linden, 2000), resulting in an immune cell-led inflammatory reaction. When endothelial damage is present, the permeability of the endothelial layer increases, allowing LDLs to pass more freely into the tunica intima. Monocytes subsequently migrate from the bloodstream with the help of adhesion to the endothelium (via adhesion receptor-mediated capture of monocytes). The monocytes undergo morphological changes whereby they flatten and squeeze between the endothelial cell layer and into the tunica intima, in a process called diapedesis (Sullivan et al., 2000). Once inside the tunica intima, monocytes become resident macrophages, producing free radicals

which interact and oxidise the LDLs present in the tunica intima. The oxidised LDLs attract and activate more macrophages, which engulf the modified LDL particles, stimulating further production of free radicals (G. W. Sullivan et al., 2000). This positive feedback loop results in accumulation of LDL particles and immune cells. Macrophages begin to engulf the modified LDLs, leading to production of foam cells, which are saturated with LDL particles. Foam cells eventually die and release their contents into the tunica intima. These contents are engulfed by other inflammatory cells. Eventually, the accumulating lipids and fragments of dead cells produce an area with a lipid core, which begins to form a plaque. The plaque hardens over time, due to accumulation of calcium salts and dead cells (Insull, 2009). If the endothelial cells covering the plaque are compromised, blood clots can form on the vessel wall – as the usual anti-coagulant properties of the endothelial cells are compromised under pathological conditions (Cooke & Tsao, 2011). Over time, ruptured areas of the plaque may jut out into the vessel lumen, forming a clot, known as a thrombus, which attaches to the wall. If the clot breaks loose from the arterial wall, and flows downstream to smaller vessels, a thromboembolism occurs, interrupting normal oxygen supply, leading to MI and stroke, dependent upon the location (Insull, 2009). The development of atherosclerosis is outlined in **Figure 2.10**.

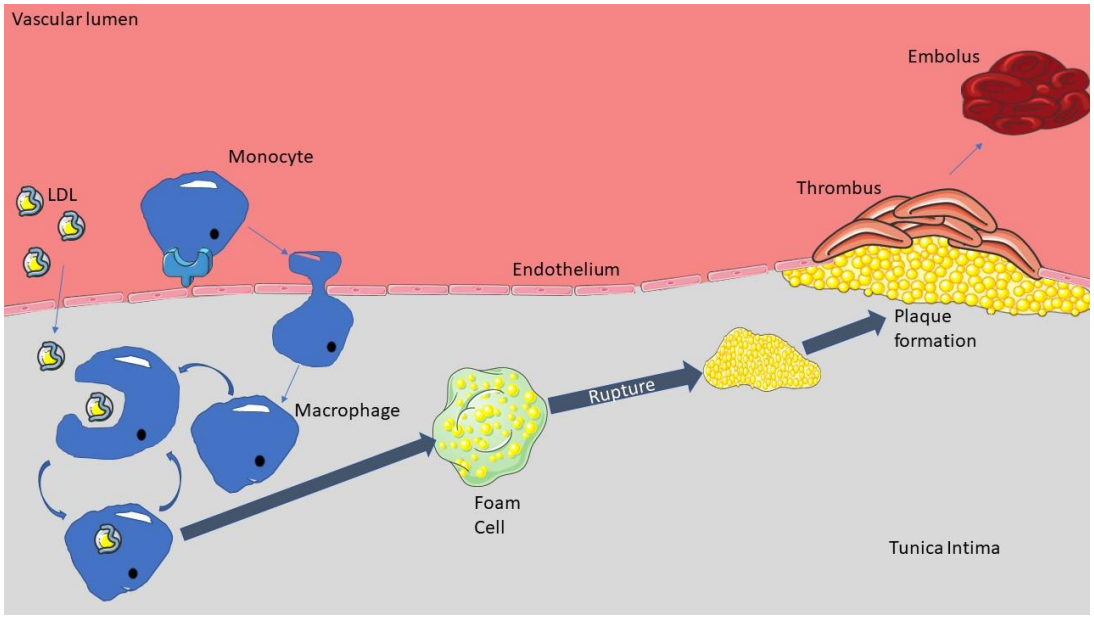

Figure 2.10. Atherosclerosis Development from LDL uptake to thromboembolism due to plaque rupture**.** *Low density lipoprotein (LDL)*

As the endothelium plays a crucial role in protecting against atherosclerotic plaque formation, thrombosis, and CVD (Mudau et al., 2012a), dysfunction of endothelial cells is probably the earliest event in the process of CVD (Verma et al., 2003). Endothelial function is a valuable prognostic tool, statistically associated with cardiovascular death, MI, and stroke (Halcox et al., 2002). Hence, assessment of the health status of the endothelium is important for early diagnosis and intervention. Macrovascular endothelial function can be quantified using FMD as an indirect measure of NO bioavailability (Al-Qaisi et al., 2008). Increasing NO bioavailability by blocking NO scavenger peroxynitrite (ONOO-) via uric acid, improves FMD, indicating that endothelial-derived NO is essential for efficient vascular function (Cassuto et al., 2014). The measure of NO bioavailability is of clinical interest due to the important role of NO in preventing atherosclerotic development as the presence of NO inhibits upregulation of adhesion molecules on the endothelial cell surface of which leukocytes bind to in the initiating steps of atherosclerotic plaque development (Mudau et al., 2012a). The adhesion molecule, ICAM-1 is pertinent to the tight binding of leukocytes to the endothelium and can be used as a marker of endothelial activation (Videm & Albrigtsen, 2008). A chronically activated endothelium leads to endothelial dysfunction (Rajendran et al., 2013). Several studies have shown endothelial dysfunction as a result of BC chemotherapy treatment (Anastasiou et al., 2017; Duquaine et al., 2003a) as well as endothelial injury (Beerepoot et al., 2004). ICAM-1 has been found to be upregulated with chemotherapy (Meijer, 2009; Mills et al., 2004) and is likely due to high levels of circulating TNFα and interleukins (Clayton et al., 2021) as illustrated in **Figure 10**. This is likely a mediating mechanism behind the cardiovascular toxicity associated with chemotherapy.

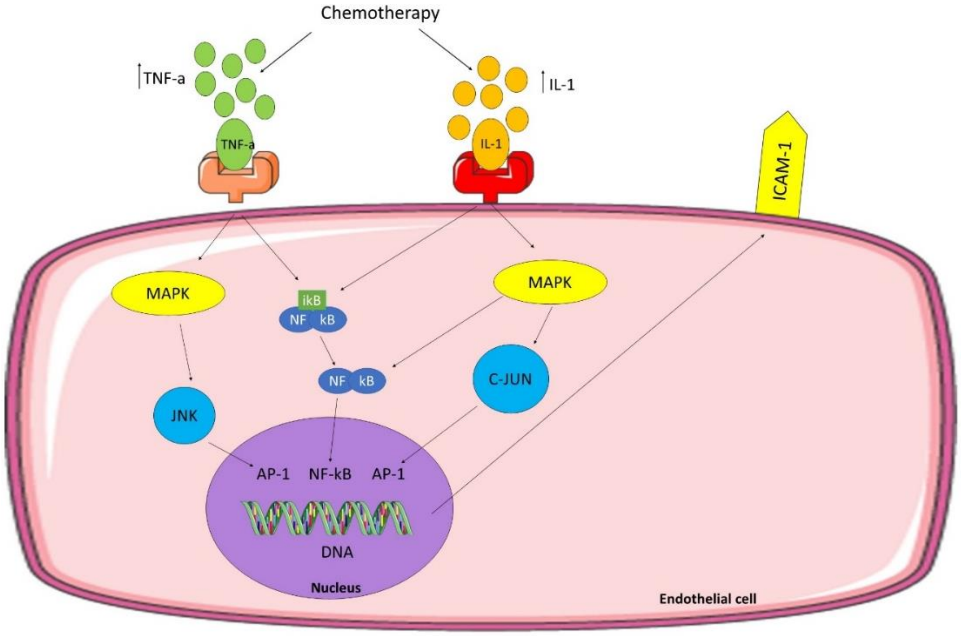

Figure 2.11. Endothelial cell activation and adhesion molecule upregulation. The binding of TNF-a to its receptor on the surface of the endothelial cell activates NF-κB signalling to activate transcription factor NF-κB. Also, TNF-a binding activates MEK kinase (MAP3K)/JNK signalling, which activates the transcription factor AP-1. The binding of IL-1 to the endothelial cell induces the direct activation of NF-κB signalling and MAPKs-induced activation of NF-κB signalling to activate NF-kB transcription factor. IL-1 activation of MAPKs also activates downstream protein c-JUN, leading to activation of the AP1 transcription factor. In any case, transcription factors NF-κB and AP-1 then activate gene transcription of inflammatory genes, leading to upregulation of surface proteins including ICAM-1.

2.2.3 Exercise Protection Against Cardiovascular Disease

It is important to protect the whole cardiovascular system against dysfunction and disease to prolong and improve life. Exercise has been historically documented as having protective effects against CVD risk, with women in increasing quintiles of energy expenditure (measured in metabolic equivalents (METs)/week) eliciting RR of cardiac events of 1.00, 0.73, 0.69, 0.68, and 0.47, respectively (Manson et al., 2002). A recent Cochrane Review concluded that exercise-based cardiac rehabilitation reduces the risk of cardiovascular mortality in CVD patients, with a RR of 0.74 compared to normal care (Anderson et al., 2016). Therefore, exercise has potential to improve cardiovascular health in other clinical populations, including BC patients to counteract toxicity of chemotherapy treatment.

Regular exercise leads to functional adaptations in cardiac structure, including physiological hypertrophy (Ellison et al., 2012), improvements in contractility

through increased Ca^{2}+ sensitivity and SERCA2a protein (responsible for re-uptake of Ca^{2}+ into sarcoplasmic reticulum during relaxation) levels, improving efficiency of both systole and diastole (Wisløff, Loennechen, Currie, Smith, & Ellingsen, 2002), and improved cellular metabolism via increased mitochondrial number (Phielix, Meex, Moonen-Kornips, Hesselink, & Schrauwen, 2010). These adaptations are responsible for exercise-induced improvements in cardiac function, leading to a higher cardiac output allowing the heart to provide more O_2 to meet increasing physiological demands during exercise. These cardiac adaptations partially contribute to improved aerobic fitness, as measured by maximum O_2 uptake ($\dot{V}O_{2max}$). Exercise interventions have been successful in increasing aerobic fitness in HF patients, with increases in $\dot{V}O_{2peak}$ of 2.6ml·kg·min^{-1} (16% increase from baseline compared with no change in usual care group) observed with 12 weeks exercise training. Exercise-induced increase in $\dot{V}O_{2peak}$ in HF patients has been attributed to improvements in LV diastolic function, measured by diastolic filling time, and reversal of atrial remodelling, assessed via echocardiography (Edelmann et al., 2011). Similarly, long-term exercise (6 months) has improved cardiac function in patients with HF, with improvements in New York Heart Association (NYHA) functional class and exercise capacity, decreased resting heart rate, increased stroke volume and LVEF, reduction in LV end diastolic diameter and total peripheral resistance during peak exercise (Hambrecht et al., 2000). Overall, exercise interventions have been successful in improving health in CVD patients, with improvements in cardiac structure and function, leading to a reduction in cardiac event risk, with a Cochrane review concluding that, exercise-based cardiac rehabilitation reduces the risk of cardiovascular mortality, with a RR of 0.74 (Anderson et al., 2016).

In addition to central cardiovascular adaptations (cardiac), exercise also stimulates significant adaptations in the vasculature. FMD positively correlates with leisure time physical activity levels (Pahkala et al., 2011). Six weeks of high intensity interval training or moderate intensity endurance training improved popliteal endothelial function, as measured by FMD (FMD pre- to post-exercise: 4.9% to 7.4%; and 5.5% to 7.4%, respectively) in healthy previously untrained subjects (Rakobowchuk et al., 2008). This is also similar for clinical populations with FMD

improving significantly from 7% to 11% in diabetic patients receiving a 12-week mixed aerobic and resistance exercise intervention compared to no change in FMD for the usual care group (Okada et al., 2010). In the 24-month follow-up period, the exercise group had a decreased number of cardiac events compared to usual care, suggesting protective effects of exercise on vascular health leading to decreased cardiac event risk (Okada et al., 2010). In HF patients, exercise interventions also enhance FMD, with a standard mean difference of 1.08 (Pearson & Smart, 2017). Compared to usual care, 4 weeks exercise training before coronary artery bypass surgery associated with a 57% higher peak flow velocity in response to acetylcholine, occurring alongside a 2- and 4-fold increase in eNOS expression and phosphorylation, respectively, suggesting enhanced endothelial function (Hambrecht et al., 2003). Improvements in vasodilatory capacity are likely due to increased bioavailability of NO through exercise-induced increase in eNOS production mediated by regular exercise-induced shear stress elevations (Rakobowchuk et al., 2008). As FMD associates with CVD (Anastasiou et al., 2017), physical activity and exercise could be a way to reduce risk of cardiovascular complications in both healthy and diseased populations (Phillips, Mahmoud, Brown, & Haus, 2015).

Furthermore, arterial compliance (elasticity of the vessel) – the ability of the vessel to respond to cardiac pulsation by expanding and decreasing in diameter – improved by 66% with both moderate and high intensity exercise (Rakobowchuk et al., 2008). An increase in compliance suggests reduction in arterial stiffness which is associated with atherosclerosis and hypertension (Arnett et al., 2000). Therefore, an exercise-induced increase in compliance represents improved vascular wall health (Koivistoinen et al., 2012) and also associates with reduced cardiovascular risk (Mitchell et al., 2010). Arterial compliance is 20-35% higher in healthy endurance-trained men, than in age-matched healthy untrained groups (Tanaka et al., 2000), adding to the evidence that exercise has beneficial effects on vascular wall health. Furthermore, a 12-week walking intervention in sedentary males increased arterial compliance by 25% (Tanaka et al., 2000). Taken together, this strongly suggests that exercise is highly vasculo-protective and the ability of

exercise to improve arterial compliance is a potential mechanism for exercise reducing the risk of CVD in healthy sedentary individuals.

Further to this, exercise may help to prevent damage to the vasculature which usually occurs in disease states, as evidenced by the 20% reduction in CECs observed with an exercise intervention in sufferers of resistant hypertension (Lopes et al., 2018). This is clinically important as endothelial damage is an early predictor and likely initiator of cardiovascular events (Cameron et al., 2016a; Werner et al., 2008). Therefore, the current literature points to exercise protection against the risk of cardiovascular events in both healthy and diseased populations.

2.3. The Potential for Exercise to Attenuate the Cardiotoxic Effects of Chemotherapy

Since exercise has improved cardiovascular health in non-cancer populations, the potential for exercise to attenuate the detrimental effects of chemotherapy on the cardiovascular system is now being explored. There is now emerging evidence that those who are physically active may have reduced risk of developing cardiovascular toxicity with chemotherapy (Nagy, Gulacsi-Bardos, Cserep, Hangody, & Forste, 2017b; J. M. Scott, Nilsen, Gupta, & Jones, 2018a; J. M. Scott, Zabor, et al., 2018). There is evidence for cardiovascular benefits of exercise interventions in cancer survivors after chemotherapy treatment, with an average of 4.6mmHg reduction in systolic BP, and 4.4mmHg reduction in diastolic BP (Sturgeon et al., 2014b). Additionally, Framingham risk scores were significantly reduced in obese women with BC after chemotherapy who exercised vs. usual care group (2% vs 13%, respectively, corresponding to an 11% decrease in predicted 10-year risk of developing CVD (Lee et al., 2019). The risk reduction was attributed to by reduced systolic blood pressure, lower LDL-cholesterol, higher HDL-cholesterol, and less diagnosis of diabetes in the exercise group compared to usual care (Lee et al., 2019). Moreover, an exercise intervention in BC survivors (3 sessions/week cycling at 60–70% $\dot{V}O_{2peak}$ for 3 months, followed by one session/week for 1 year) resulted in improved reactive hyperaemia index (Giallauria et al., 2016). Physical activity, for example walking, has been effective in reducing arterial stiffness, measured by pulse wave velocity, in BC patients (Bucciarelli et al., 2017).

Arterial stiffness is partly modulated by endothelial dysfunction (Donley et al., 2014). Exercise-induced improvements in endothelial function may contribute to improved arterial function via shear stress-induced upregulation of eNOS expression and phosphorylation, resulting in enhanced arterial compliance in response to increased flow (Donley et al., 2014). Therefore, the endothelium may play a central role in reducing arterial stiffness via exercise training, highlighting the importance of the endothelium in governing overall vascular health. Furthermore, self-reported physical activity inversely associates with cardiovascular events, with BC women following exercise guidelines after treatment having a 23% reduction in cardiovascular events from 2 months to 15 years post-treatment, compared to

those who undertook less physical activity (Jones et al., 2016). These findings are promising for reducing risk of cardiovascular events after treatment. However, it is most advantageous to prevent toxicity occurring in the first instance, as this would increase treatment tolerance, completion and prevent necessary attenuation of treatment dosage/delayed cycles. However, evidence for cardiovascular benefits of exercising before and during their chemotherapy treatment is scarcer.

2.3.1 In-Human Studies of Exercise-Induced Cardiovascular Protection Before Chemotherapy

Exercise prior to chemotherapy may be a strategy to reduce cardiotoxicity of treatment by preconditioning the cardiovascular system to improve resilience against damage/insult (Newcomer, Thijssen, & Green, 2011; Palmefors, DuttaRoy, Rundqvist, & Börjesson, 2014a; Randers et al., 2013) before exposure to toxic drugs. However, evidence from in-human studies is limited. Thirteen BC patients performed a single acute exercise session (30 minutes of vigorous-intensity exercise) 24 hours prior to the first chemotherapy treatment, resulting in decreased systemic vascular resistance, with chemotherapy alone having no significant effect on vascular resistance (Kirkham et al., 2017). Furthermore, increased ejection fraction and systolic strain rate occurred from pre- to post-chemotherapy in the exercise group with no change in these outcome measures for the chemotherapy alone group. Relative to control, the exercise group also had a significantly lower N-terminal pro-brain natriuretic peptide (NT-proBNP; common diagnostic marker for cardiac dysfunction) and was associated with a 46% risk reduction in HF incidence (Kirkham et al., 2017). Although this is the only in-human study of its kind, these findings are promising.

More evidence for exercise prehabilitation having protective cardiac effects before chemotherapy treatment comes from observational studies of individuals with high cardiorespiratory fitness (CRF) compared with individuals with low CRF. A recent study has found that those with higher aerobic capacity (as measured by $\dot{V}O_{2peak}$) before chemotherapy treatment associated with a less severe anthracycline-induced cardiac fibrosis (Kirkham et al., 2020). Therefore, those with high CRF are protected against chemotherapy-induced cardiac side effects, which may be associated with physical activity levels. However, it also must be noted that $\dot{V}O_{2max}$

has a significant genetic influence (Joyner, 2001), and therefore this effect may not be entirely due to physical activity and/or exercise.

2.3.2 In-Human Studies of Exercise-Induced Cardiovascular Protection During Chemotherapy

Exercising during chemotherapy for BC has also been found to have a positive impact on the cardiovascular system, with a meta-analysis finding an average reduction in systolic blood pressure by 4.4mmHg and diastolic blood pressure by 1.3mmHg from pre- to post-intervention (Sturgeon et al., 2014b). This is clinically relevant as a 20mmHg decreased systolic BP associates with a 20% decreased annual risk of CVD (Lewington et al., 2002). Therefore, a 4.4mmHg reduction in systolic blood pressure may indicate a 4.4% reduction in CV risk. Furthermore, exercise during chemotherapy has also been found to prevent the usual reduction in cardiovascular fitness/CRF induced by chemotherapy treatment (Mijwel et al., 2018). More specifically, chronic exercise interventions during chemotherapy had been associated with a 2.8ml·kg·min^{-1} increase in $\dot{V}O_{2max}$ compared with no change in usual care control group (Scott et al., 2018). Improvements in aerobic fitness are clinically important as a 3.5ml·kg·min^{-1} increase in $\dot{V}O_{2max}$ is associated with a 12-17% decrease in future all-cause mortality risk in cancer patients (Jones et al., 2010). However, one study showed LVEF was still reduced in those undergoing an exercise intervention during chemotherapy, despite attenuation of $\dot{V}O_{2max}$ detriments (Howden et al., 2019). Another study included biomarkers of cardiac toxicity in their analyses of the effects of 16-weeks of resistance training plus HIIT, moderate-intensity aerobic plus HIIT, or usual care during chemotherapy (Ansund et al., 2021). At 1-year follow-up, NT-pro-BNP was lower in the exercise groups compared to usual care, suggesting a protective effect of exercise on the heart in the longer term. However, there was no effect of exercise on plasma high sensitivity cardiac troponin T, with increases observed in all groups post-intervention, suggesting similar cardiotoxicity with or without exercise. Interestingly, after 2-years, $\dot{V}O_{2peak}$ decreased in those with high cardiac troponin T (> 10 ng·ml^{-1}) post-intervention and high Nt-pro-BNP (> 100 ng·ml^{-1}) at 1-year follow up, suggesting that these biomarkers are predictive of reduced functional

capacity. The resistance training plus HIIT group had less patients eliciting these elevated biomarkers and a lower risk for reduced $\dot{V}O_{2peak}$, compared to usual care (OR 0.20; 95% CI = 0.06–0.73). Therefore, in this trial, a chronic exercise intervention – particularly resistance training plus HIIT – during chemotherapy preserved cardiovascular function ($\dot{V}O_{2peak}$) and attenuated the long-term cardiac damage associated with chemotherapy (Ansund et al., 2021).

Beneficial effects of exercise during chemotherapy for BC have also been observed for vascular health and function, with an increase in FMD from pre-to post-doxorubicin-cyclophosphamide chemotherapy in the exercise group (3 sessions/week cycling at 60-100%$\dot{V}O_{2peak}$ for 30-45min) compared to no change in FMD in the usual care group (Jones et al., 2013), however, this was not a statistically significant increase (p = 0.07). More promising results were found with a cycling intervention alongside anthracycline chemotherapy or usual care only, whereby post-intervention, brachial FMD increased by 4.3% with exercise compared to the 7.15% reduction in FMD in the usual care group (Lee, Kang, et al., 2019a), which is a clinically meaningful finding, as a 1% increase in FMD is associated with an 8-13% reduced risk of CVD and *vice versa* (Inaba, Chen, & Bergmann, 2010).

Furthermore, an exercise intervention attenuated the usual increase in carotid intima media thickness (thickness of the inner two layers of the carotid artery - measure of atherosclerotic development) with chemotherapy treatment, suggesting a protective effect of exercise against atherosclerosis, likely due to endothelial protection (Lee et al., 2019a). Hence, focussing on vascular health and circulating factors may be important for preventing early-stage cardiovascular toxicity, often manifesting as vascular dysfunction. Interestingly, a recent study has found that changes in haemodynamics are attributed to detrimental changes in cardiac output. Doxorubicin treatment alone reduced haematocrit, reducing blood viscosity, in turn reducing mean arterial pressure. Mean arterial pressure must be maintained at basal levels for endothelial health and function as shear stress is responsible for endothelial production of potent vasodilator NO and its subsequent anti-thrombotic properties vessel (Wedgwood et al., 2012).Therefore, the reduction in arterial pressure was compensated for by the heart which increased cardiac

output in order to maintain vascular function (Kirkham et al., 2020). However, a chronically increased cardiac output increases strain on the myocardium, leading to cardiomyopathy and HF (Gibbs et al., 2000), as detailed in **Chapter 2.2.1**. With exercise training during doxorubicin treatment, there was a favourably decreased vessel lumen size – a compensatory mechanism to prevent the usual reduction in systemic vascular resistance in response to chemotherapy-induced reduction in haematocrit (Kirkham et al., 2020). Preventing the reduction in systemic vascular resistance with exercise training prevented the usual compensatory increase in cardiac output and likely the subsequent strain on the heart (Kirkham et al., 2020). This is the first study which has investigated the effect of haemodynamic changes and the subsequent vascular consequences which have the potential to contribute/alleviate to cardiotoxicity of chemotherapy, and therefore, further research is required to expand these findings.

As well as macrovascular function, muscle capillarisation has also improved with a 16-week exercise training intervention during chemotherapy treatment. Standard chemotherapy results in reduced muscle capillary number, however, exercise training attenuated this decline (Mijwel et al., 2018). This is important as a decline in skeletal muscle microvascular structure and function has been proposed to precede macrovascular impairments (Krentz, Clough, & Byrne, 2009) and reduces the ability of the skeletal muscle to meet its metabolic demands, contributing to the development of CVD (Clerk et al., 2006; Vincent, Barrett, Lindner, Clark, & Rattigan, 2003; Wallis et al., 2002). Taken together, findings indicate that exercise protects both micro- and macrovascular health from the negative effects of chemotherapy

2.3.3 *In Vivo* Models of Exercise Before Chemotherapy to Attenuate Cardiovascular Toxicity

The majority of evidence for the benefits of exercise prehabilitation comes from rodent studies. Healthy non-cancer rats which performed a single aerobic exercise bout (60 minutes, 25m/min treadmill running) 24 hours before doxorubicin treatment ($15mg \cdot kg^{-1}$) had significantly higher end-systolic pressure and LV developed pressure, compared with sedentary BC rats receiving doxorubicin ($15mg \cdot kg^{-1}$), indicating exercise-induced cardiovascular protection (Wonders et al., 2008). Similarly, chronic resistance training (consisting of a raised-caged model,

which forces rats to assume an erect bipedal stance whilst eating and drinking) 12 weeks before doxorubicin treatment (12.5mg·kg^{-1}) is also cardio-protective in non-cancer rats, with reductions in mortality from 27% in usual care control group to 13% in exercise group, observed alongside preserved cardiac function, likely due to prevention of changes in contractile proteins (Pfannenstiel & Hayward, 2018). Both voluntary wheel-running (moderate intensity) and endurance treadmill running (high intensity) 5-weeks pre- and during treatment prevented oxidative stress and reduction in function of mitochondrial bioenergetics in the hearts of healthy male Sprague-Dawley rats injected with doxorubicin (2mg·kg^{-1} per week for 7 weeks) (Inês Marques-Aleixo et al., 2015). Furthermore, exercise prevented doxorubicin-induced increases in mitochondrial susceptibility to increased permeability and apoptotic signalling within cardiac tissue (Marques-Aleixo et al., 2018). A recent meta-analysis concluded that the key mechanism of exercise protection against cardiotoxicity is due to reduced oxidative stress and doxorubicin accumulation within cardiomyocytes (Naaktgeboren et al., 2021).

As with in-human studies, chemotherapy-induced vascular toxicity has had less research than cardiac toxicity in animal models of exercise during chemotherapy treatment. However, some preliminary evidence has demonstrated that, despite no significant detriment in vasodilatory capacity with 5-FU exposure, 8, but not 4 weeks of exercise training (treadmill running at 20–25 m·min^{-1}, 15% grade, 30 min·d^{-1}, 5 d·wk^{-1}) prior to exposure to 5-FU was associated with enhanced endothelium-dependent vasodilation in BC rats (injected with 25 mg·kg^{-1} N-methyl-N-nitrosourea resulting in mammary tumour formation), suggesting that exercise may need to be of a more chronic nature to elicit protective effects on the vasculature (Hayward et al., 2004). Taken together, the evidence for exercise 'prehabilitation' in animal models is promising, with maintenance of cardiac function, likely due to preserved contractile properties, reduced oxidative stress, protection of mitochondrial health, reduced apoptosis, and improved endothelial function.

2.3.4 *In Vivo* Models of Exercise During Chemotherapy to Attenuate Cardiovascular Toxicity

Animal models provide an invaluable opportunity to further explore the potential for exercise to alleviate cardiovascular toxicity of chemotherapy and have been utilised in several research studies. Exercise training during chemotherapy preserves cardiac function in rodents. Performing 45 minutes of treadmill walking/day at 12m/s, 5 days/week for 12 weeks prevented both early and late doxorubicin-induced cardiotoxicity and increased recovery from an ischemic event in juvenile mice (Wang, Chandra, & Kleinerman, 2021). In female mice, treadmill running (5 days/week, 18m/min, 45 mins/session for 8 weeks or 3 times/week moderate intensity aerobic exercise for 4 weeks) attenuated the increases in LV systolic volumes and reduction in fractional shortening, LVEF and aerobic capacity induced by doxorubicin (cumulative dose: 24mg·kg^{-1}) (Jones et al., 2011; Yang et al., 2020), and this is likely through inhibition of cardiac fibrosis and inflammation (H. L. Yang et al., 2020). Wheel running during and following doxorubicin treatment preserved maximal mitral and aortic blood flow velocities and left ventricular developed pressure in rats receiving doxorubicin (cumulative dose: 15 mg·kg^{-1}) (Hydock et al., 2012). Rats performing voluntary wheel running during doxorubicin treatment had more desirable distributions of contractile proteins (preserving normal β-myosin heavy chain expression) than sedentary rats (increased β-myosin heavy chain expression which associates with cardiac dysfunction (Hydock et al., 2011)) receiving the same doxorubicin regime (cumulative dose: 15 mg·kg^{-1}) (Hydock et al., 2012; Hydock, Wonders, Chicco, Schneider, & Hayward, 2005). Moreover, low intensity exercise training in combination with doxorubicin treatment (cumulative dose: 15 mg·kg^{-1}) attenuated LV dysfunction, increased expression of antioxidant enzymes, and prevented apoptotic signal activation (Chicco, Hydock, Schneider, & Hayward, 2006). This is important as induction of apoptosis is a mediating mechanism behind toxicity of all FEC-T drugs, as highlighted in **section 2.1.2** (Chighizola et al., 2011; Gligorov & Lotz, 2004; Longley et al., 2003; Stěrba et al., 2013). Similarly, in a rat model of doxorubicin toxicity (whereby physiological concentrations of chemotherapy were used), moderate treadmill running for 45 min 5 days per week for 8 weeks prevented increases in

arterial stiffness and systolic dysfunction (Dolinsky et al., 2013). In addition, mitochondrial function was preserved, most likely due to the alleviated oxidative stress with exercise training, as indicated by increased antioxidant manganese superoxide dismutase (assessed by Western Blot).

Protective effects of exercise are also observed in vascular function. Exercise training during doxorubicin treatment resulted in between-group difference in endothelium-independent but not endothelium-dependent vasodilation, with impaired vascular smooth muscle function in the sedentary group, contrasting no change from pre- to post-exposure in the exercise group (Matsuura et al., 2010). This suggests that exercise preserves vascular function, but the mechanism is not via increased NO bioavailability but instead via preserved smooth muscle relaxation properties. This contrasts with findings from Hayward et al. (2004) who found that NO-dependent vasodilation was also statistically different between exercise and sedentary groups exposed to 5-FU. The differences in findings may be due to the different chemotherapy regimens utilised.

Interestingly, exercise during chemotherapy in mice, increased tumour microvessel density, vessel maturity and perfusion, and reduced intra-tumoral hypoxia, compared with sedentary controls. This associated with significantly reduced tumour growth and a 1.4-fold increase in apoptosis (Betof et al., 2015). Hence, exercise may protect from vascular toxicity by increasing capillarization, which in turn can be beneficial for efficacy of the chemotherapy treatment itself. Overall, *in vivo* models have provided promising initial evidence for cardiovascular protection, with preservation of cardiac function likely mediated through maintenance of contractile proteins, reduction in apoptotic signalling, and increased antioxidant availability, as well as improved endothelial function and capillarisation.

2.3.5 Potential Mechanisms for Protective Effects of Exercise During Chemotherapy to Prevent Toxicity

Evidence is now emerging that exercise during chemotherapy can ameliorate cardiovascular toxicity of treatment, as demonstrated by maintained systolic and diastolic function. The potential mechanisms behind this include a reduction of cardiovascular risk factors with exercise training interventions (Lee, Kang, et al.,

2019a). It is well documented that risk factors such as pre-existing CVD, hypertension, hypercholesterolemia, physical inactivity, diabetes, and obesity are strong predictors of cardiotoxicity of chemotherapy (Barrett-Lee et al., 2009; Gavila et al., 2017; Jones et al., 2007; Yancik et al., 2001). Therefore, reducing these risk factors via exercise is a plausible reason for reduction of cardiotoxicity risk with treatment. Furthermore, newly proposed studies investigating markers of troponin, NTpro-BNP, and inflammatory mediators, should elucidate mechanisms of cardiac toxicity but currently there is little or no data available to produce any conclusions.

Since there are a limited number of in-human studies investigating exercise protection against toxicity of chemotherapy on the cardiovascular system, evidence from animal models can be used to determine possible mechanisms at a cellular and molecular level. Exercise training in combination with chemotherapy attenuated cardiac dysfunction via a reduction in apoptotic signal activation (Chicco et al., 2006). Apoptosis of cardiomyocytes is inhibited, protecting the overall structure of the heart, preserving cardiac function. The reduction in apoptosis may be due to a reduction in oxidative stress, which is a mediator for apoptotic signalling cascade. Exercise during doxorubicin treatment increased antioxidant glutathione peroxidase expression (Chicco et al., 2006). Enhanced antioxidant defences may be responsible for a reduction in oxidative stress, further reducing apoptotic signalling. Exercise also prevented apoptotic signalling from the mitochondria. The mitochondria are the organelles responsible for production of energy in the form of adenosine-triphosphate, allowing the myocardium to perform cellular functions (Marques-Aleixo et al., 2018). Studies observed an exercise-induced protection of normal levels of oxidative phosphorylation (Inês Marques-Aleixo et al., 2015) and the expression of proteins responsible for the process (Jones et al., 2011), in turn preserving function of mitochondrial bioenergetics and cellular function, reducing the likelihood of initiation of mitochondrial-induced signalling of cellular apoptosis.

As well as reduced apoptosis, there is now evidence for chronic exercise-induced preservation of cardiac function and prevention of the α- to β-myosin heavy chain shift that occurs with doxorubicin exposure (Hydock et al., 2012, 2005; Pfannenstiel & Hayward, 2018). The relative expression of α- to β-myosin heavy chain isotypes

correlate with the contractile velocity of the myocardium. With exercise, this expression ratio is maintained, allowing the myocardium to function normally. This is an interesting finding which gives strong support to there being a protective effect of exercise against chemotherapy-induced cardiac toxicity in BC.

With regards to vascular health, the maintenance of FMD and capillarisation observed with exercise training during chemotherapy treatment is promising for BC patients (Mijwel et al., 2018). FMD is endothelium dependent (Hayward et al., 2004), which means that there is an endothelial-derived increase in NO bioavailability due to the enzyme, eNOS. This is promising for reducing risk of cardiovascular events as NO has anti-atherogenic properties, reducing the risk of atherosclerosis development. However, another study found that the exercise did not ameliorate deficits in endothelium-dependent vasodilation and in fact, only improved endothelial-independent vasodilation (Matsuura et al., 2010). Of course, the ability of the smooth muscle to respond to vasodilatory stimuli (such as NO), contributes to the levels of vasodilation observed. Therefore, these findings suggests that it is exercise-induced adaptations to the vascular smooth muscle, likely mechanical adaptations, which has improved vasodilation in this case. This means that the smooth muscle cells are able to react to more efficiently to the increase in shear stress induced by the sudden return of blood flow, without the requirements for increased NO bioavailability. These contrasting findings means further investigation is required into the mechanisms behind improved vascular function.

2.3.6 Serological Alterations with Physical Activity/Exercise which Potentially Contribute to Reduced Vascular Toxicity

Regular physical activity results in serological adaptations which may protect against chemotherapy-induced vascular damage individuals (Conti et al., 2012a; Petersen & Pedersen, 2005; Sapp, Landers-Ramos, Shill, Springer, & Hagberg, 2020). Interestingly, serum from physically active individuals contains more antioxidants than that of inactive individuals (Conti et al., 2012a), potentially protecting against chemotherapy-induced oxidative stress (Bouzid, Filaire, Matran, Robin, & Fabre, 2018). Furthermore, high leisure-time physical activity associates with higher levels of anti-inflammatory markers, including interleukin-1 and -10 and reductions in pro-

inflammatory cytokines such as TNF-α (Petersen & Pedersen, 2005). This is likely to protect against further environmental stress when endothelial cells are exposed to chemotherapy drugs (Joussen et al., 2009). Furthermore, metabolic factors, including improved glucose and HDL-cholesterol levels are likely to contribute to reduced apoptosis (Durham, Chathely, & Trigatti, 2018; Frias et al., 2010; Kodama et al., 2007; Leskinen et al., 2013; Risso, Mercuri, Quagliaro, Damante, & Ceriello, 2001). It is also important to consider that physically active individuals have higher circulating VEGF (Sapp et al., 2020), which is highly important for endothelial cell health, as this growth factor is key for proliferative and migratory processes (Conti et al., 2012b; Sapp et al., 2020).

Acute exercise results in thousands of molecular changes in metabolic, hormonal, and immunological factors which stimulate or repress cell growth and migration, cardiovascular pathway signalling, and oxidative stress pathways (Contrepois et al., 2020; Naylor et al., 2020). This has the potential to influence vascular toxicity of chemotherapy as, for example, increased circulating VEGF levels at 10-minutes post-exercise (Wahl et al., 2011) is likely to stimulate the migrate and proliferation of endothelial cells (Grunewald et al., 2021; Kimura et al., 2006; Palazón-Bru, Hernández-Lozano, & Gil-Guillén, 2021), alongside suppression of apoptotic pathways (Yang et al., 2012) which may counteract the chemotherapy-induced stimulation of apoptosis (Ching et al., 2021; Erdbruegger et al., 2006; Sales et al., 2019; Winther et al., 2016). Furthermore, a single exercise session lowers circulating blood glucose (Lira et al., 2017), which also has the potential to prevent endothelial cell apoptosis (Altannavch, Roubalová, Kučera, & Anděl, 2004; Risso et al., 2001). More metabolic consequences of acute exercise include reduction in LDL-cholesterol, alongside an increase in circulating HDL-cholesterol (Greene, Martin, & Crouse, 2012). Both of which have potential to protect vascular health as LDL-cholesterol plays a key role in atherosclerotic plaque development (Sullivan et al., 2000); and HDL-cholesterol has been found to elicit protection against chemotherapy-induced apoptosis in cardiomyocytes (Durham, Chathely, Mak, et al., 2018; Durham, Kluck, Mak, Deng, & Trigatti, 2019). Acute exercise also elicits time-dependent increases and subsequent decreases in circulating inflammatory markers, including IL-1 and TNFα (Orange, Jordan, & Saxton, 2020) which stimulate

endothelial adhesion molecule expression (Bernot, Peiretti, Canault, Juhan-Vague, & Nalbone, 2005). Overall, acute exercise serological alterations have potential importance for vascular protection against chemotherapy exposure.

There are also potentially important longer-term serological alterations observed with exercise interventions. Exercise interventions have been found to increase circulating growth factors, including VEGF which promotes cellular migration (Grunewald et al., 2021; Kimura et al., 2006; Palazón-Bru et al., 2021); as well as increasing circulating HDL-cholesterol which has the potential to protect against chemotherapy-induced apoptosis (Durham, Chathely, Mak, et al., 2018; Durham, Chathely, & Trigatti, 2018; Durham et al., 2019; Kimura et al., 2003). Similar to acute exercise, exercise interventions also elicit reductions in resting blood glucose levels which potentially protects vascular endothelial cells against further environmental damage when exposed to chemotherapy (Scott et al., 2019). There are also higher levels of antioxidants and subsequently lower levels of oxidative stress from pre- to post-exercise interventions (Carlsohn et al., 2008), which is likely to counteract the increased ROS generation induced by chemotherapy exposure (Clayton et al., 2020; Clayton, Hutton, et al., 2021; Stěrba et al., 2013). **Figure 2.12.** provides an overarching hypothesis of the protective effects of physical activity/exercise on the vascular endothelium when exposed to FEC-T chemotherapy.

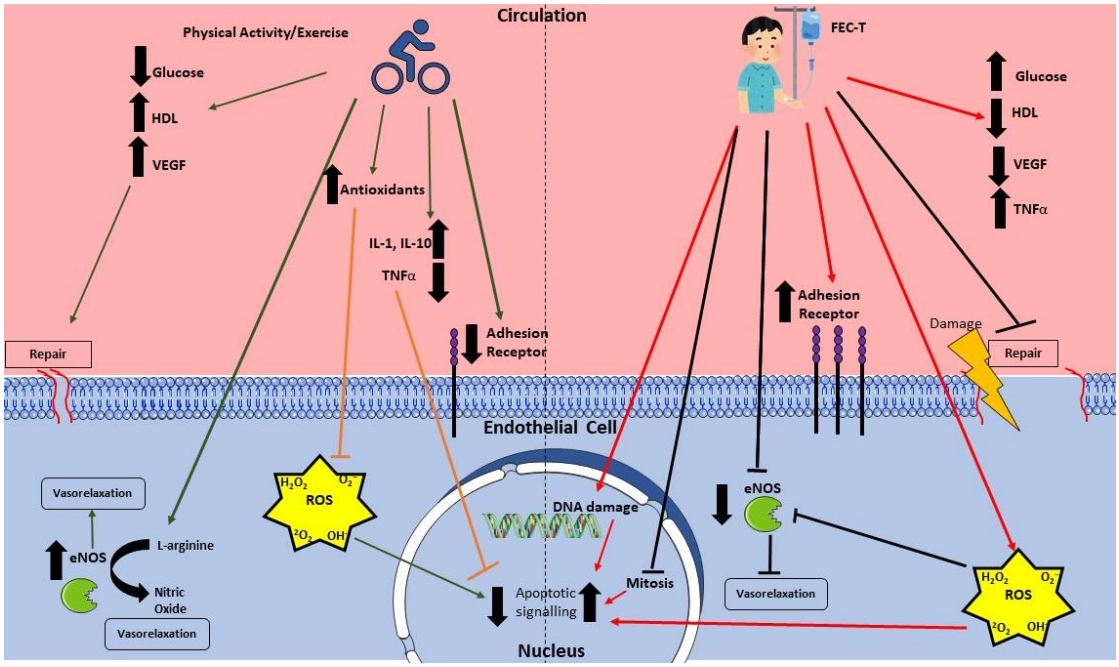

Figure 2.12. Mechanisms of chemotherapy toxicity on the endothelium, including endothelial cell activation and apoptotic signalling; and the potential counteractive effects of physical activity/exercise through improved circulatory profile. *Vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), high density lipoprotein-cholesterol (HDL)*

2.3.7 *In Vitro* Models of Exercise During Chemotherapy to Attenuate Cardiovascular Toxicity

Currently, there are no known *in vitro* models of potential exercise-induced protection against the toxicity of chemotherapy on the cardiovascular system. Research into this would allow potential mechanisms to be elucidated at a cellular and molecular level, without the ethical and implementational difficulties involved in human or animal models. *In vitro* models allow for elucidation of mechanisms for the potential exercise protection against toxicity, allows identification of target pathways, and to determine whether exercise effects are systemic or localised. Recently, exercise oncology studies have successfully utilised serological preconditioning models to investigate the effects of acute exercise on tumour cell growth (Orange et al., 2020). These models have provided valuable insight into the mechanisms of exercise protection against cancer progression. Furthermore, serological conditioning models have been used for non-cancer studies of exercise and endothelial cell health (Wahl et al., 2014). Despite being effectively used in other research areas, *in vitro* models are an under-utilised in the field of exercise and cardiovascular toxicity. Therefore, it should be recommended that *ex vivo*

models are utilised in future research as these provide a potentially valuable means for researching exercise and chemotherapy exposure on endothelial cells (Orange et al., 2020; Wahl et al., 2014).

2.4. Conclusion

Overall, current evidence is beginning to suggest that there is a cardioprotective effect of exercise during chemotherapy treatment for BC, with animal models showing strong evidence with plausible mechanisms. However, there are some human studies finding amelioration of cardiac dysfunction and some not observing this protection, with reasons for contrasting findings not investigated but likely due to between-study differences in exercise and treatment regimes. The mechanisms for exercise cardio-protection during chemotherapy have not yet been investigated in human studies. This leaves assumptions to be drawn from other studies in non-chemotherapy treated populations and animal models of chemotherapy treatment. Regarding vascular toxicity, there is insufficient evidence to provide a strong conclusion regarding exercise protection of the vasculature in BC, but promising results have been found in preliminary studies. Therefore, more investigation is required into the potential protection of exercise during chemotherapy treatment for BC on the heart and vasculature and the possible mechanisms responsible for this. Perhaps *in vitro* studies should be pursued to achieve these aims. Moreover, most studies investigated doxorubicin. However, use of this drug is now declining due to the increase in use of the less toxic anthracycline, epirubicin. Evidence shows that epirubicin is still toxic to the cardiovascular system. Further research is required into the mechanisms and possible prevention of epirubicin-induced toxicity, as well the other drugs involved in FEC-T treatment for BC. Additionally, current research focusses on cardiac effects and there is a paucity of research investigating vascular toxicity of chemotherapy, despite the vasculature being the first point of contact of the chemotherapy drugs upon infusion, and despite vascular damage and dysfunction preceding most cardiac dysfunction. Assessing the vasculature may give us a better understanding of the underlying mechanisms of chemotherapy-induced toxicity and is a potential method for early detection and better management of chemotherapy toxicity.

2.5. Hypothesis

The primary goal of this PhD was to test the hypotheses that (1) FEC-T chemotherapy drugs exert toxicity on endothelial cells; (2) serum conditioning from healthy habitually physical active females can alleviate endothelial cell toxicity of BC chemotherapy treatment when compared to serum from healthy inactive controls; that (3) preconditioning endothelial cells with serum taken after acute exercise even in those previously untrained can alleviate vascular toxicity of treatment; and that (4) a long-term exercise intervention in 'at risk' females can alleviate vascular endothelial toxicity of treatment via serum preconditioning. We tested our hypotheses through a four-phase process utilizing *in vitro* models of endothelial exposure to chemotherapy using an *ex vivo* conditioned serum approach to assess the potentially protective effects of physical activity/exercise protection, which is summarized below and explained in detail in the subsequent chapters.

1. Firstly, an *in vitro* model of endothelial exposure to chemotherapy was developed using human umbilical vascular endothelial cells (HUVEC) and human coronary artery endothelial cells (HCAEC) to determine the toxic effects of chemotherapy exposure using flow cytometric assessment of cell damage and dysfunction, and analysis of wound repair.
2. Endothelial cells were then preconditioned with serum from physically active and sedentary individuals before chemotherapy exposure to compare potential serum protection of habitual physical activity vs inactivity by analyzing the same outcome measures as before.
3. Then, a similar study was conducted investigating the protective effects of an acute exercise session in the inactive participants, using the same *ex vivo* serum conditioning and chemotherapy exposure approach in HCAECs, with the same methods of endothelial cell analysis.
4. The last step of our study was to determine whether serum collected after an exercise intervention had the potential to protect against the usual toxicity of FEC-T chemotherapy exposure in endothelial cells using the same *ex vivo* approach in the HCAECs *in vitro* model.

Chapter 3
General Methodology and Materials

The following section provides details of the methods common to more than one study of this thesis. Additional techniques and methods used in separate studies are described in relevant chapters.

3.1 *In Human* Methods and Materials

3.1.1 Participant Recruitment and Ethics

Participants for study *Chapter 4 and 5* were recruited using a recruitment poster displayed both around Edinburgh Napier University, Sighthill campus, and online on social media sites (**Appendix 1**). Participants were then selected based on the following inclusion criteria: healthy females, Caucasian, 18-35 years old, BMI 18-24.9kg·m^2, currently taking contraceptive medication. Participants were provided with written and verbal explanations of the aims, procedures, and risks relating to the study, and of their right to withdraw from the study at any stage, before providing written informed consent (**Appendix 2&3**). They were given a minimum of 24 hours from the date of receiving full information of the study to decide whether or not to participate. Participants were excluded for presence of diabetes, neurological conditions, hypertension, CVD, and smoking status. All data was stored on password-protected computers, and participant anonymity was maintained in accordance with GDPR guidelines. Ethical approval was granted from Edinburgh Napier University ethics committee. All participants provided informed signed consent before participation in this study.

For *Chapter 7*, serum was obtained from participants who partook in a study led by Liverpool John Moore's University (Hesketh, 2020). The parent study was approved by the Liverpool Central NHS Research Ethics Committee (approval reference no. 17/NW/0042) and conformed to the Declaration of Helsinki. The secondary use of the serum, as acellular, non-genetic material was approved by the Liverpool Central NHS Research Ethics Committee, Liverpool John Moore's University, and by the Edinburgh Napier University Research Ethics and Governance committee.

3.1.2 Participant Measurements

Height and body mass were recorded using stadiometer and scales, respectively, on the first visit to determine BMI. After 5 minutes rest in supine position, heart rate was recorded throughout using a chest strap monitor (H9 Beat, Polar, Finland) and blood pressure was recorded using an automated blood pressure cuff (M2, Omron, Japan) inflated around the dominant arm with participant in supine position.

3.1.3 Questionnaires

The AHA/ACSM health screening questionnaire (Cardinal & Balady, 1999) (**Appendix 4**) and The Physical Activity Readiness Questionnaire (PAR-Q) (Warburton et al., 2011) (**Appendix 5**) was completed by participants to ensure safety and suitability for exercise participation. Participants also completed The International Physical Activity Questionnaire (IPAQ) (Hagströmer, Oja, & Sjöström, 2006) (**Appendix 6**). The IPAQ was completed to classify participants as "active" or "inactive" according to the WHO guidelines (>150 minutes/week moderate-vigorous physical activity (MVPA) = "active", <150 minutes/week MVPA = "inactive" (Mensink, 1999)). However, for the purpose of this thesis, 'inactive' has been classed as <120 minutes/week MVPA to ensure sufficient differentiation between groups.

3.1.4 Phlebotomy

Participants were instructed to consume 300ml water 1 hour before blood draw to ensure similar hydration statuses and maximise plasma yield (Benozzi, Unger, Campion, & Pennacchiotti, 2018). Venous blood was collected from the antecubital vein in serum separation tubes (BD Biosciences, USA) (10ml) Phlebotomy occurred before any exercise testing so as acute exercise effects did not interfere with sampling. The first 2ml in in serum separation tubes (BD Biosciences, USA) were discarded as vascular injury present in blood. Samples were allowed to clot at room temperature for 30 minutes, centrifuged at 1500 x *g* for 10 minutes at 4^{o}C, and serum subsequently removed. Once all serum was collected, samples were pooled based on exercise training status, creating one active and one inactive sample. Serum samples were aliquoted into 1ml aliquots to avoid freeze-thaw cycles as samples, frozen at -20°C and stored for later analysis. For acute exercise study only,

whole blood samples were collected 15 minutes after exercise and analysed as above.

3.1.5 Assessment of Peak Oxygen Consumption ($\dot{V}O_2$peak)

$\dot{V}O_{2peak}$, the gold standard assessment of CRF was measured in participants in study *Chapter 5 and 6*. On the day of testing, participants arrived at 9am and were fasted for 12 hours, having participated in no exercise and no consumption of caffeine, drugs, or alcohol within the last 24-hours. The exercise tests were performed on a treadmill (Woodway, ERGO ELG-55, Weil am Rhein, Germany). Participants walked on the treadmill for 2 minutes as a warm-up and to allow them to adjust and familiarise with the equipment. The incremental exercise test subsequently commenced, with the treadmill initially starting at 8km·hr^{-1} and increasing by 1km·hr^{-1} every minute. Participants were verbally encouraged to exercise to volitional exhaustion, defined as intolerable dyspnoea. $\dot{V}O_{2peak}$ was measured using a breath-by-breath gas analyser (Cortex Metalyzer 3B, Leipzig, Germany), which was calibrated before use and metabolic analysis software (MetaSoft Studio, v5.8.6 SR1, Cortex, Leipzig, Germany). An appropriately fitting face mask was worn by the participants, alongside a chest strap heart rate monitor (H9 Beat, Polar, Kempele, Finland). $\dot{V}O_{2peak}$ was determined as the average $\dot{V}O_2$ of the last 30 seconds of the test or as the peak whereby the $\dot{V}O_2$ was consistent for 4 consecutive breaths (Barker, Williams, Jones, & Armstrong, 2011; Rossiter, Kowalchuk, & Whipp, 2006).

For study 6, an incremental exercise test to exhaustion was performed on an electromagnetically braked cycle ergometer. Briefly, patients began cycling at 25W for 3 minutes, and workload increased by 35W every 3 minutes until volitional exhaustion. $\dot{V}O_{2peak}$ was determined using an online gas collection system (Moxus Modular Oxygen Uptake System, AEI Technologies, Illinois, USA). $\dot{V}O_{2peak}$ was defined as the highest $\dot{V}O_2$ achieved over a 15 second recording period.

3.2 *In Vitro* Methods and Materials

3.2.1 Cell Culture Techniques

3.2.1.1 Thawing and Seeding (HUVECs and HCAECs)

In order to investigate the *in vitro* effects of chemotherapy on endothelial cells and the potential attenuation of these effects by serological preconditioning, passage 1 human umbilical vascular endothelial cells (HUVECs) were sourced (Thermofisher Scientific, Waltham, USA, product code: C0035C) and cultured using Endothelial Cell Growth Medium (ECGM; Sigma-Aldrich, Gillingham, UK, product code: 211-500) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Thermofisher Scientific, Waltham, USA), 50µg/ml penicillin and 50µg/ml streptomycin. Passage 2 unpooled human coronary artery endothelial cells (HCAECs) (product code: 300-05A, Sigma-Aldrich, Gillingham, UK) were cultured using Meso-Endothelial Cell Growth Medium (MEGM; Sigma-Aldrich, Gillingham, UK, product code: 212-500) supplemented with 10% (v/v) FBS (FBS) (Gibco), 50µg/ml penicillin and 50µg/ml streptomycin. For initial seeding, a 1.5ml cryovial containing 500,000 cells were partially thawed in 37^{o}C water bath and fully thawed with addition of 1ml of warm medium, pipetting up and down to disperse cells into suspension. The solution was then removed from the cryovial and placed in a 15ml falcon tube (BD Bioscience, Franklin Lakes, USA), with 10ml media, and centrifuged for 5 minutes at 250 x *g*. The supernatant was carefully discarded to remove any dimethyl sulphoxide (DMSO) from the freezing mix. The cell pellet was resuspended in 10ml medium and transferred to a T-75 flask (Corning, New York, USA), equating to seeding density of 500,000 cells per T-75 flask. The T-75 flask was incubated at 37^{o}C, 5% CO_2. The following day, to remove any dead or floating cells from the flask, all media was removed, cells washed with 10ml phosphate buffer serum (PBS; Invitrogen, Thermofisher, Waltham, USA, product code: 003002), 10ml fresh ECGM (Sigma-Aldrich, Gillingham, UK, product code: 211-500) or MEGM (Sigma-Aldrich, Gillingham, UK, product code: 212-500) was added, and flasks re-incubated at 37^{o}C, 5% CO_2. This process is illustrated in Figure **3.1**.

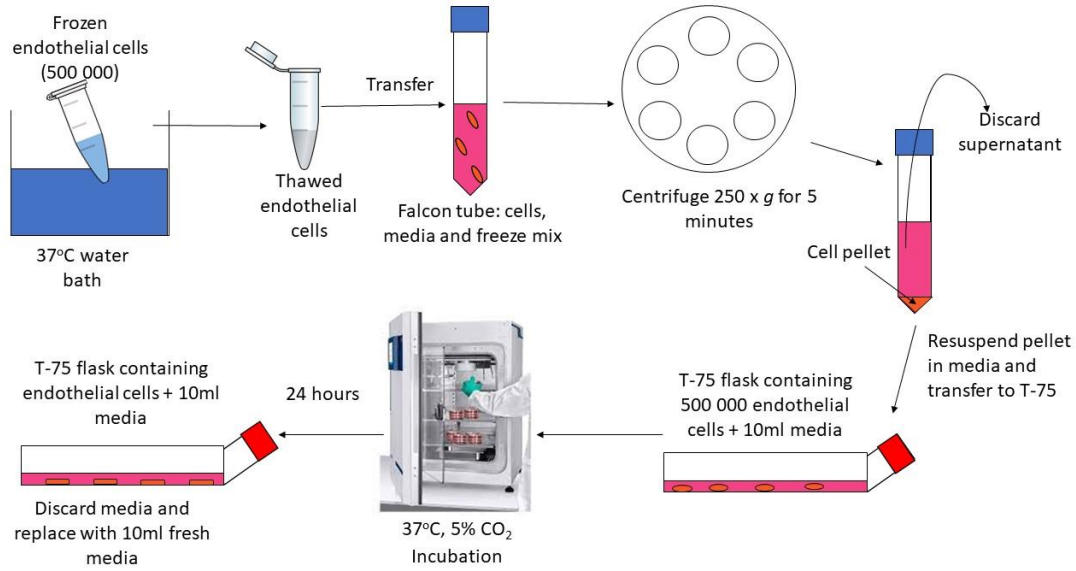

Figure 3.1. Endothelial cell thawing and seeding protocol.

Flasks were imaged daily using Primovert Axiocam ERc5s microscope (ZEISS, Germany) at magnifications 4x, 10x, and 20x (**Figure 3.2**).

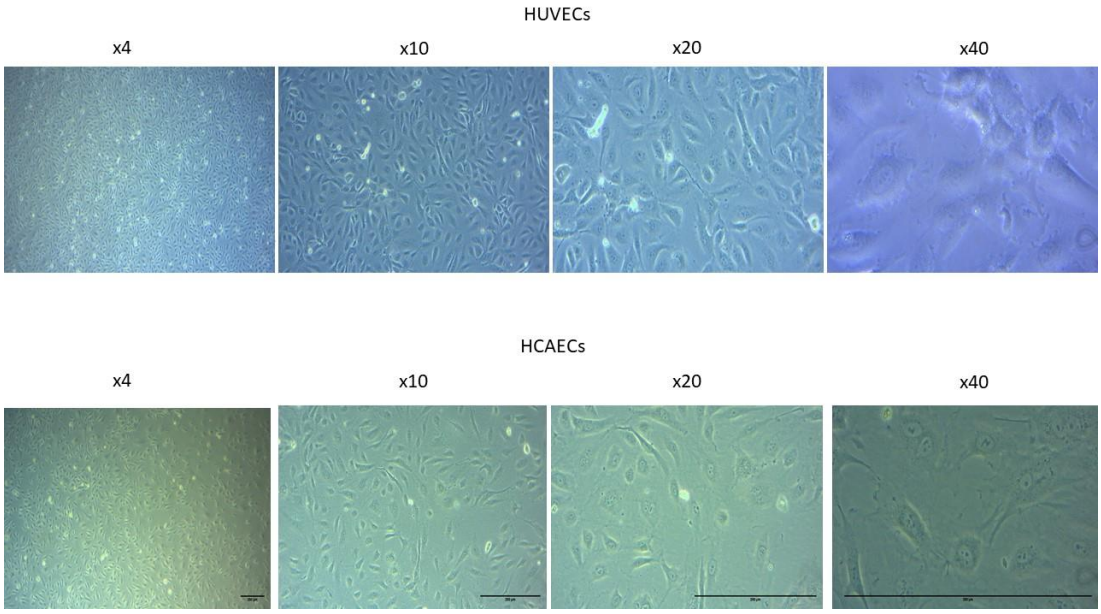

Figure 3.2. Microscope imaging of HUVECs (A-D) and HCAECs (A-H) at x4 (A, E), x10 (B, F), x20 (C, G) and x40 (D, H) magnifications. *Scale bars are 250µm.*

3.2.1.2 Subculturing (Passaging) of Endothelial Cells

At 50% confluency, media was removed and 10ml fresh warm medium was added to flasks, every 48 hours. At 60% confluency, this process was repeated every 24 hours, until 80% confluency where the cells were passaged using the following process: all media was removed from T-75 flasks, and cells washed with 5ml NaCl. Cells were treated with 2ml of trypsin/EDTA (Gibco, Thermofisher Scientific, Waltham, USA, product code: 15400054) solution for 1-2 minutes, until cells become round and detached from the surface of the flask. 5ml media was then added to the flask to neutralize the trypsin. The cells were transferred from the T-75 flask into a 50ml Falcon tube (BD Bioscience, Franklin Lakes, USA). The Falcon tube was centrifuged at 250 x *g* at 22°C for 5 minutes. The supernatant was carefully discarded, and the cell pellet resuspended in 10ml warm medium. The solution was then split into new sterile T-75 flasks and mixed with fresh media at a ratio of 1:3. This subculturing process is illustrated in **Figure 3.3**. The newly passaged flasks were then incubated at 37°C, 5% CO_2, imaged and given fresh media until 80% confluent, as previously described.

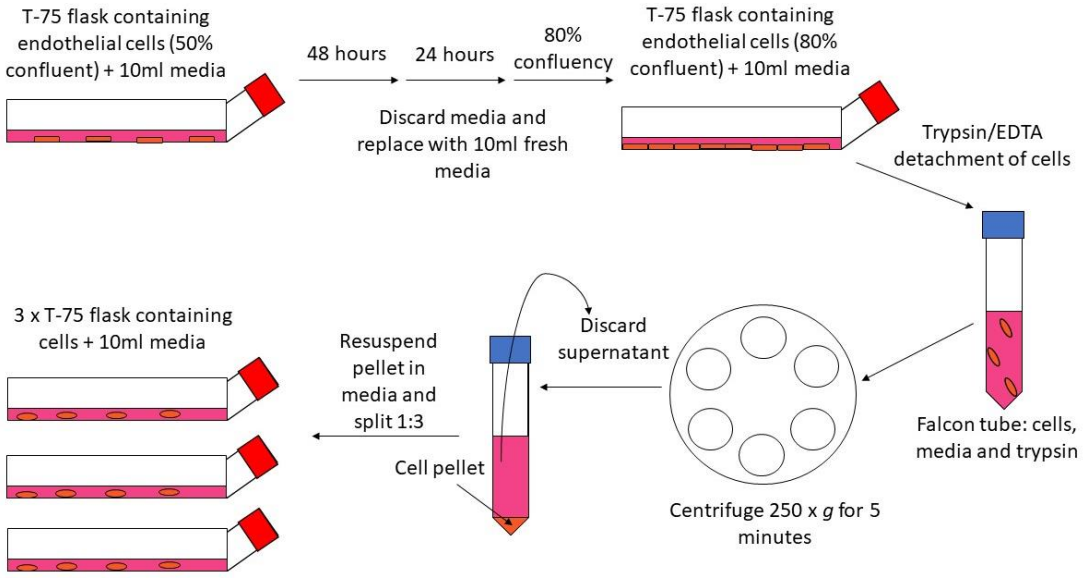

Figure 3.3. Protocol for subculturing (passaging) of endothelial cells at a 1:3 ratio.

3.2.1.3 Freezing of the Endothelial Cells

During passages, excess cells were frozen and stored for later use. The freezing process involved pre-cooling the freezing container (Thermofisher Scientific, Waltham, USA) with isopropanol (VWR chemicals, USA, product code: 20842.367) at 4°C. Freezing mix (95:5, media:DMSO) was also pre-cooled on ice for 15 minutes. Endothelial cell monolayers were detached from confluent T-75 flasks and centrifuged to create a cell pellet, using the above procedures. The cell pellet was then resuspended in 1ml freezing mix plus 1ml medium. The suspension was aliquoted (4 x 0.5ml) into 1ml cryovials which were then placed into the pre-cooled freezing container and stored in -80^{o}C freezer for 24 hours. After 24-hours, cryovials were transferred to liquid nitrogen for long-term storage. When thawing these from liquid nitrogen, the same protocol was used as in the initial seeding procedure.

3.2.1.4 Seeding Endothelial Cells for Experiments

On the 3rd-8th passage, cells were detached from their flasks at ~80% confluency, and centrifuged, as previously described. The cell pellet was resuspended in 1ml media and a cell counting process was followed. Cells were counted by mixing 10µl of the suspension with 10µl of trypan blue stain, and 10µl of this 1:1 solution was then pipetted onto a cell counting slide and analysed using Primovert Axiocam ERc5s microscope (ZEISS, Germany). HUVECs were seeded in a 12-well plate at 100,000 cells/well and 1ml ECGM added to each well. HCAECs were plated in a 24-well plate (50,000 cells/well) with 500µl of MEGM. All plates were incubated for 24 hours at 37^{o}C (5% CO_2) to allow cells to adhere fully to wells. This process is illustrated in **Figure 3.4**.

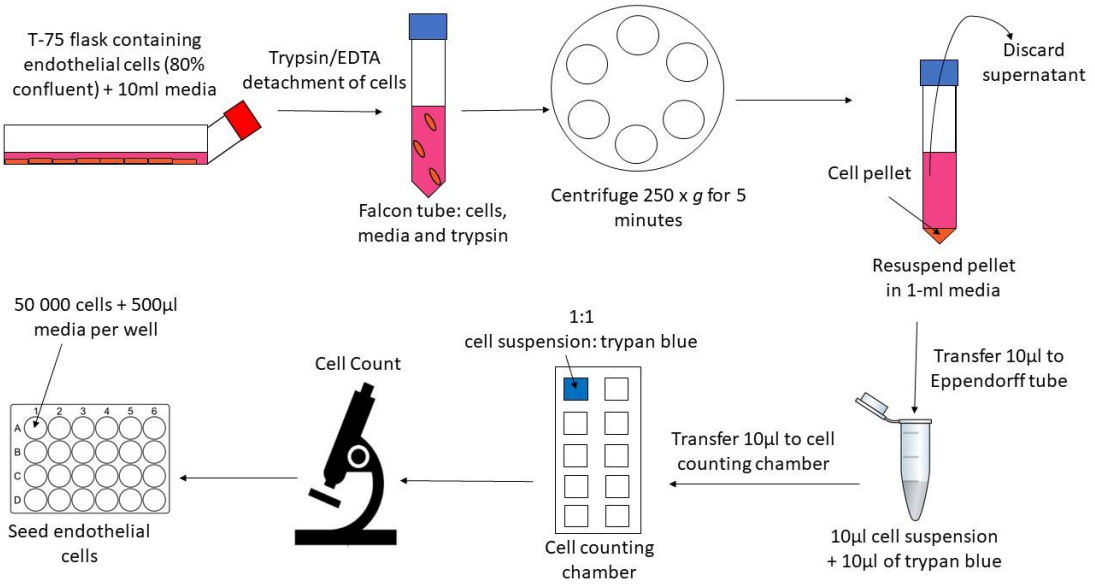

Figure 3.4. Endothelial cell seeding into 24-well plate for experiments.

3.2.1.5 Chemotherapy Exposure

DMSO (Honeywell, UK, product code: 51779) was used as a vehicle to dilute 5-fluorouracil (product code: ab142387; Abcam, UK), epirubicin hydrochloride (product code: ab142100; Abcam, UK), cyclophosphamide (Abcam, UK, product code: ab141240) and docetaxel (Abcam, UK, product code: ab141248) to their appropriate concentrations. All chemotherapy drugs are presented in **Table 3.1**. Solutions were then added to cells at the correct volumes to corresponding wells.

Table 3.1. Chemicals required for chemotherapy exposure experiments and for phospho-eNOS antibody validation experiment.

Chemical	Quantity	Company
Dimethyl sulfoxide (DMSO)	1 litre	Honeywell, UK
5-fluorouracil	5g	Abcam, UK
Epirubicin Hydrochloride	5mg	Abcam, UK
Cyclophosphamide	50mg	Abcam, UK
Docetaxel	10mg	Abcam, UK

For HUVEC experiments, FEC-T was investigated firstly as single drugs and then in

combination (FEC), as commonly prescribed in early-stage BC treatment. For HCAECs, FEC combined, and docetaxel single treatments were used only. Drug concentrations were based on the serum concentration found in patients after exposure to each of these drugs (5-FU: 1.5µM (Reigner et al., 2003); epirubicin: 0.006µM (Danesi et al., 2002); cyclophosphamide: 38µM (Adams, Johnson, & Murray, 2014); and docetaxel: 6µM (Hurria et al., 2006)). A concentration 50% above and 50% below the physiological level was included to allow a potential dose-response effect to be investigated, and an additional well with media only was used as a no drug control. Cells were incubated at 37°C in 5% CO_2 until washing time-points. Drugs were washed off at time-points corresponding to the literature findings when serum levels of each drug were diminished. At these time points (5-FU: 3- hours (Reigner et al., 2003); epirubicin: 1- hour (Danesi et al., 2002); cyclophosphamide: 12-hours (Adams et al., 2014); docetaxel: 24-hours (Hurria et al., 2006); FEC: 4-hours), all media was removed from each well, cells washed with 400µl NaCl and 1000µl fresh ECGM added to the wells before re-incubating at 37°C in 5% CO_2 until the appropriate individual analysis time-points. This protocol can be visualised in **Figure 3.5**.

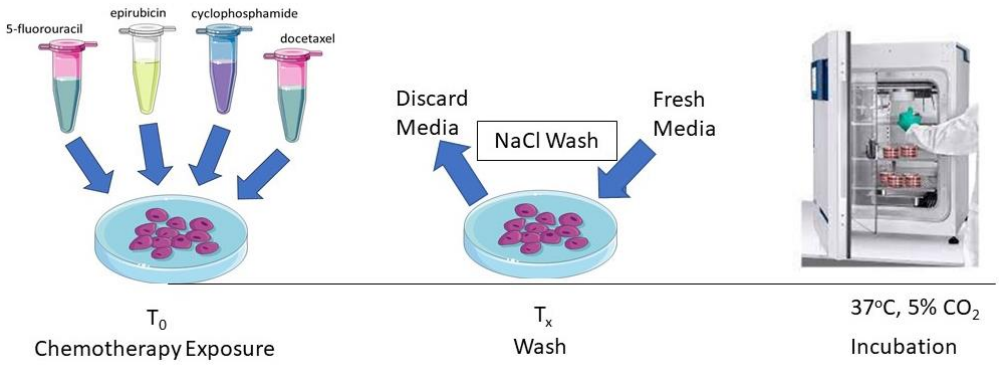

Figure 3.5. Schematic protocol of chemotherapy exposure, washing and re-incubation.

3.2.1.6 Serological Preconditioning of Endothelial Cells

To determine whether exercise has a protective effect on the chemotherapy exposure, cells were pre-conditioned with human serum for 24 hours. Twenty four hours after the initial cell plating, media was removed and replaced with fresh media mixed with 5% pooled serum (Cogan et al., 2019). Plates were re-incubated

at 37°C in 5% CO_2 for 24-hours, before exposure to physiological concentrations of FEC-T drugs, as in chemotherapy-only wells, with media volumes adjusted to accommodate the additional 5% serum. This procedure was used for all 3 studies (*Chapter 4*: habitual physical activity; *Chapter 5:* acute exercise; *Chapter 6*: exercise training intervention). This serum preconditioning protocol can be visualised in **Figure 3.6**. Cells were imaged using Primovert Axiocam ERc5s microscope (ZEISS, Germany) at magnifications 4x, 10x, and 20x, to determine any visual changes in cell morphology prior to detachment.

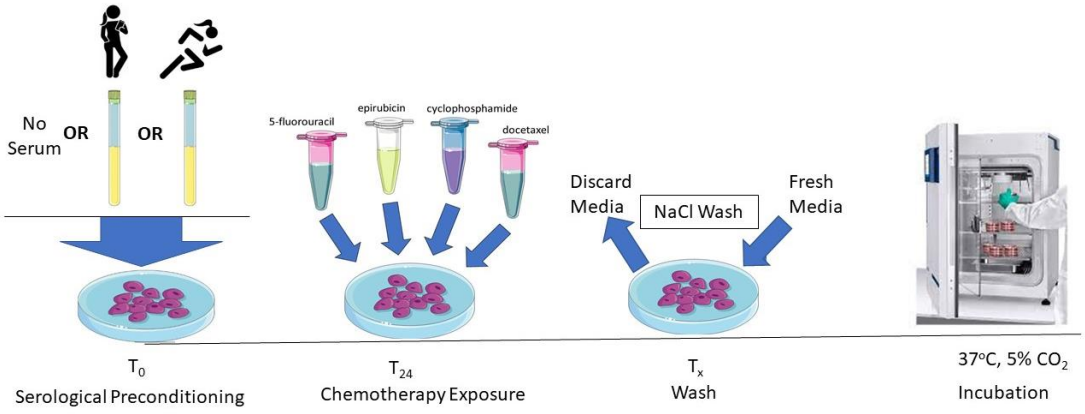

Figure 3.6. Schematic protocol of serum preconditioning, chemotherapy exposure, washing and re-incubation.

3.2.2. Flow Cytometric Analysis of Endothelial Cell Apoptosis, Caspase-3 Activity, Adhesion Receptor Expression and eNOS Expression

3.2.2.1 Multicolour Flow Cytometry

Flow cytometry is a technique by which populations of interest (in this case, endothelial cells) are detected and measured based on physical and chemical properties. A fluidic sample containing endothelial cells was injected into the 12-colour flow cytometer (FACS Celesta, BD Biosciences, Franklin Lakes, USA). Endothelial cells entered the fluidics channel in single file, moving past a laser beam, creating scattered and fluorescent light based on size, granularity and whether the cell was carrying fluorescent molecules (Macey, 2007). Generally, endothelial cells were stained by monoclonal antibodies labelled with fluorescent dyes. The flow cytometer (FACS Celesta, BD Biosciences, Franklin Lakes, USA) uses

lasers to detect the fluorescent dyes which emit different wavelengths of light, allowing for detection of both extracellular and intracellular antigens and receptors, permitting characterisation of cellular properties, including apoptotic state, eNOS, and adhesion molecule expression. Each fluorescent marker is excited at a specific wavelength of light to differentiate them when using multiple markers, allowing for detection of multiple antibodies simultaneously (Macey, 2007). The detection of scattered light emission is converted to a voltage pulse (directly proportional to the scattered light) and was displayed on the computer software (FACSDiva 6.0 Software, BD Bioscience, Franklin Lakes, USA) as histogram plots, with the amount of forward scattered light on the x-axis. The general process of flow cytometry is displayed in **Figure 3.7**.

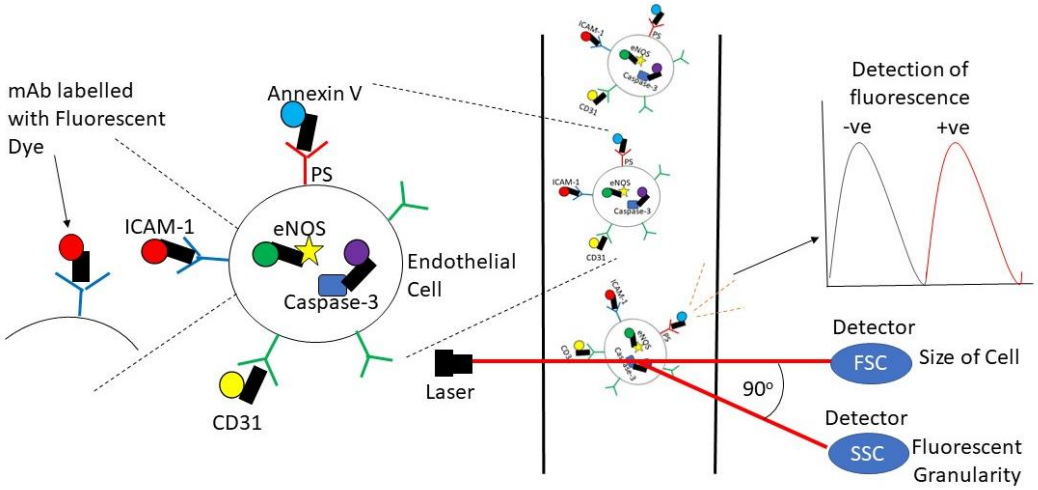

Figure 3.7. General flow cytometry technique for analysis of endothelial cell properties using monoclonal antibodies labelled with fluorescence dyes.

3.2.2.2 Sample Preparation for Analysis

The individual drugs were analysed at the most potent time-points, according to the literature. These time-points are as follows: 5-FU: 3 and 12 hours; epirubicin: 4 and 12 hours; cyclophosphamide: 3 and 6 hours (Chow & Loo, 2003); and docetaxel: 24 and 48 hours (Morse, Gray, Payne, & Gillies, 2005); FEC: 4 and 12 hours, based on our own findings. At the respective time-points, media was removed, and cells were washed with 400µl NaCl, and 200µl trypsin-EDTA (Gibco, Thermofisher Scientific, Waltham, USA, product code: 15400054) solution was used for cell detachment. For HUVEC experiments, half the solutions (300µl) were

transferred to their corresponding wells in a 96 V-bottom plate and centrifuged at 300 x *g* for 3 minutes at 21°C. The supernatant was carefully discarded and the other 300µl solution from 12-well plate transferred to the 96-well plate and centrifuged as before. The supernatant was carefully discarded, and cells resuspended in 100µl phosphate buffer serum (Invitrogen, Thermofisher, Waltham, USA, product code: 003002) or Annexin V binding buffer (Biolegend, UK, product code: 422201) for analysis by flow cytometry. For HCAEC experiments, as cultured in 24-well plates, media was removed, and cells were washed with 200µl NaCl, and 100µl trypsin-EDTA (Gibco, Thermofisher Scientific, Waltham, USA, product code: 15400054) solution was used for cell detachment. All of the solution (300µl) was transferred to the corresponding wells in a 96 V-bottom plate and centrifuged at 300x *g* for 3 minutes at 21°C (as HCAECs cultured in 24-well plates). For adhesion molecule analysis, media was removed and HCAECs were washed with 100µl PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002) ready for analysis (without detachment at this stage).

3.2.2.3 Flow Cytometry Analysis

The effects of chemotherapy on endothelial cell viability, apoptosis, adhesion molecules and eNOS content were determined by flow cytometry using a panel of monoclonal antibodies (mAb). The optimal working antibody concentrations were determined by titration experiments using PBMCs from whole blood samples and endothelial cell samples. Briefly, cells were singularly stained with increasing concentrations of the mAbs, creating a concentration curve to determine the optimal concentration for fluorescent staining. For anti-CD31-FITC (endothelial cell marker; BioLegend, USA, product code: 303104), anti-phospho-eNOS-PE (Biorbyt, UK, product code: orb124607), anti-Annexin V-PerCP-Cy5.5 (BD Biosciences, Franklin Lakes, USA, product code: 561431), and anti-caspase-3-BV650 antibodies (BD Biosciences, Franklin Lakes, USA, product code: 564096), fluorescent saturation was observed at 1.5µl, 2µl, 2µl, and 2µl per 500 000 cells, respectively. Hence, these concentrations were used for subsequent experiments. For anti-eNOS-PE (Miltenyi Biotec, Bergisch Gladbach, Germany, product code: 130-106-787) and fixable viability dye-V450 (BD Biosciences, Franklin Lakes, USA, product code: 562247),

there was a continuous increase in fluorescent intensity as the antibody concentration increased and hence, the supplier recommended concentration was used for these mAbs (1µl per 1 million cells).

Using data from the titration studies 50,000 HUVECs were incubated with anti-CD31-FITC (endothelial cell marker; BioLegend, USA, product code: 303104), fixable viability stain-V450 (BD Biosciences, Franklin Lakes, USA, product code: 562247), anti-Annexin V-PerCP-Cy (BD Biosciences, Franklin Lakes, USA, product code: 561431) and incubated for 30 minutes at 4°C. Likewise, 50 000 HCAECs were incubated with anti-CD31-FITC (product code: 303104; BioLegend, USA) and anti-Annexin V-PerCP-Cy5.5 (product code: 561431; BD Biosciences, Franklin Lakes, USA) and incubated for 30 minutes at 4°C. The cells were washed twice with 100µl perm wash buffer (BD Biosciences, Franklin Lakes, USA, product code: 554714). Endothelial cells were then permeabilised for intracellular staining, using 100µl fixation and permeabilization solution (BD Biosciences, Franklin Lakes, USA, product code: 554714) and incubated for 20 minutes at 4°C. Cells were washed twice with 100µl wash buffer (BD Biosciences, Franklin Lakes, USA, product code: 554714). HUVECs were then incubated with anti-cleaved caspase-3-BV650 (BD Biosciences, Franklin Lakes, USA, product code: 564096). With FEC combined treatment only, anti-eNOS-PE (Miltenyi Biotec, Bergisch Gladbach, Germany, product code: 130-106-787) and anti-phospho-eNOS-PE (Biorbyt, UK, product code: orb124607) mAbs were also added (to separate wells as on same fluorophore). HCAECs were incubated with anti-cleaved caspase-3-V450 (product code: 560627; BD Biosciences, USA) and anti-eNOS-PE (product code: 130-106-787; Miltenyi Biotec, Bergisch Gladbach, Germany). Endothelial cells were then incubated for a further 30 minutes at 4°C, washed twice with 100µl perm wash buffer (BD Biosciences, USA, product code: 554714), resuspended in 200µl PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002).

For analysis of adhesion molecules, HCAECs were fixed and stained with antibodies in-plate before detachment. HCAEC monolayers were fixed with 100µl 0.5% formaldehyde at 4°C for 5 minutes, washed with 100µl PBS and re-incubated in 100µl PBS (0.1% bovine serum albumin) with anti-CD31-FITC mAb (BioLegend, USA product code: 303104) and anti-ICAM-1/CD54-AlexaFluor 488 mAb (BioLegend, UK,

product code: 322713) for 30 min at 4°C. The HCAEC monolayer was washed with 100µl PBS before detachment with 100µl accutase (Gibco, Thermofisher Scientific, Waltham, USA) solution. Cells were transferred to a 96 v-bottom plate and centrifuged at 300G for 3 minutes at 21°C. Supernatant was discarded and cells resuspended in 200µl PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002) for analysis by flow cytometry. All materials and antibodies required for flow cytometry sample preparation and analysis are presented in **Table 3.2** and **3.3**, respectively.

Table 3.2. Materials required for flow cytometry sample preparation

Consumable	Quantity	Company
Annexin V Ca^{2+} buffer (10x solution)	50mL (diluted in NaCl)	BD Bioscience, USA
Fixation/Permeabilization solution	125ml	BD Bioscience, USA
BD Perm/Wash buffer (10x solution)	100ml (diluted in dH_2O)	BD Bioscience, USA
Trypsin/EDTA (10x solution)	Diluted in NaCl	Gibco, Thermofisher Scientific, USA
Accutase	500ml	Gibco, StemPro, Thermofisher Scientific, USA
Phosphate Buffer Saline	Tablets in dH_2O	Invitrogen, Thermofisher, USA

Table 3.3. Antibodies required for analysis by flow cytometry

Antibody	Conjugation	Quantity	Concentration	Company

anti-CD31	FITC	300µl	1µl/ml	BioLegend, USA
anti-Annexin V	PerCP-Cy5.5	200µl	0.5µl/ml	BD Bioscience, USA
anti-Cleaved caspase-3	BV650	200µl	2µl/ml	BD Bioscience, USA
Fixable Viability Stain	V450	100µl	1µl/ml	BD Bioscience, USA
anti-eNOS	PE	300µl	2µl/ml	Miltenyi Biotec, Germany
anti-phospho-eNOS	PE	100µl	2µl/ml	Biorbyt, UK
anti-CD54 (ICAM-1)	Alexa Fluor® 488	100µl	2.5µl/ml	BioLegend, USA

All samples were analysed using High Throughput System (HTS) on a 12-colour flow cytometer (FACS Celesta, BD Biosciences, USA). This process of sample preparation for analysis by flow cytometry is illustrated in **Figure 3.8**.

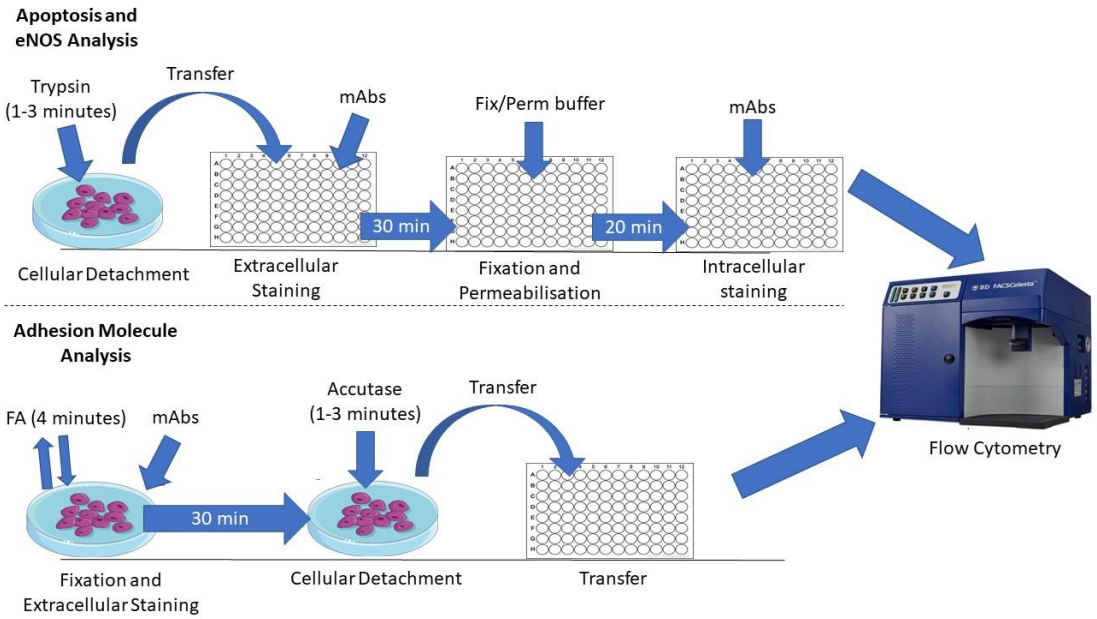

Figure 3.8. Exemplar of endothelial cell sample preparation and staining for analysis by flow cytometry, comparing apoptosis/eNOS panel and adhesion molecule panel.

3.2.2.4 High Throughput System (HTS) Setup and Data Acquisition

Flow cytometer laser setup and calibration were performed using CS&T beads (BD Biosciences, USA) every week to ensure reliable data acquisition between experiments. Data were acquired using FACSDiva 6.0 Software (BD Bioscience). For apoptosis and eNOS panel, fluorescence compensation was performed by using Anti-Mouse Ig compensation beads (BD CompBeads Set, BD Biosciences, USA) and endothelial cells, prior to sample acquisition. This automatically determined the mathematical compensation for any fluorescent spill-over between lasers and the methodology for this is described in detail in *Chapter 3.2.2.7*. For sample acquisition, the cells were set as stopping gate of 10,000 events on the High-Throughput System (HTS). HTS was set to consistently standardized parameters: flow rate µL/sec = 2.0; sample volume = 100µL; mix volume = 100µL; mix speed = 75µL/sec; number of mixes = 5; wash volume = 300µL.

3.2.2.5 Flow Cytometry Voltages

Firstly, voltages were selected based on the best visual representation of the events. Voltages for all stained fluorophores were selected based on a clear negative and positive population (all voltages are presented in **Tables 3.4-7**).

Table 3.4 & 3.5. Voltages settings for lasers for HUVEC panel 1 & 2

Parameter	Voltage
FSC	374
SSC	250
V450	390
BV650	560
PerCP-Cy5.5	700
FITC	495

Parameter	Voltage
FSC	374
SSC	250
V450	390
BV650	560
PerCP-Cy5.5	690
FITC	495
PE	470

Table 3.6 & 3.7. Voltages settings for lasers for HCAEC panel 1 & 2.

Parameter	Voltage
FSC	341
SSC	231
Alex Fluor 488	390
Pacific Blue	560
PE	700

Parameter	Voltage
FSC	341
SSC	231
V450	300
PE	400
PerCP-Cy5.5	549
FITC	456

3.2.2.6 Flow Cytometry Gating Strategy

Firstly, dot plots were created to determine the size and morphology of all events, allowing selection of the population of interest. A plot of forward scatter-area and -height was then created, gated on the population of interest. From this plot, singlets were selected. All subsequent plots were gated on singlets to remove any potential doublets from data analysis. FITC-CD31$^+$ expression confirmed endothelial cell identity. For HUVEC experiments, once gated on CD31$^+$ cells, dot plots and histograms of viability-V450, of cleaved caspase-3-BV650, Annexin V-PerCP-Cy, eNOS-PE, and Phospho-eNOS-PE could be visualised, and data interpreted. Representative gating strategy is shown in **Figure 3.9**. For HCAECs experiments, once gated on CD31$^+$ cells, dot plots and histograms of cleaved caspase-3-V450, annexin V-PerCP-Cy5.5, eNOS-PE, and ICAM-AlexaFluor 488, could be visualised, and the data interpreted. Representative gating strategy is shown in **Figure 3.9**, using unstained cells as a negative control sample; stained chemotherapy exposed cells as positive controls for non-viable, annexin V, and caspase-3 plots; and healthy stained endothelial cells as eNOS and ICAM-1 positive controls.

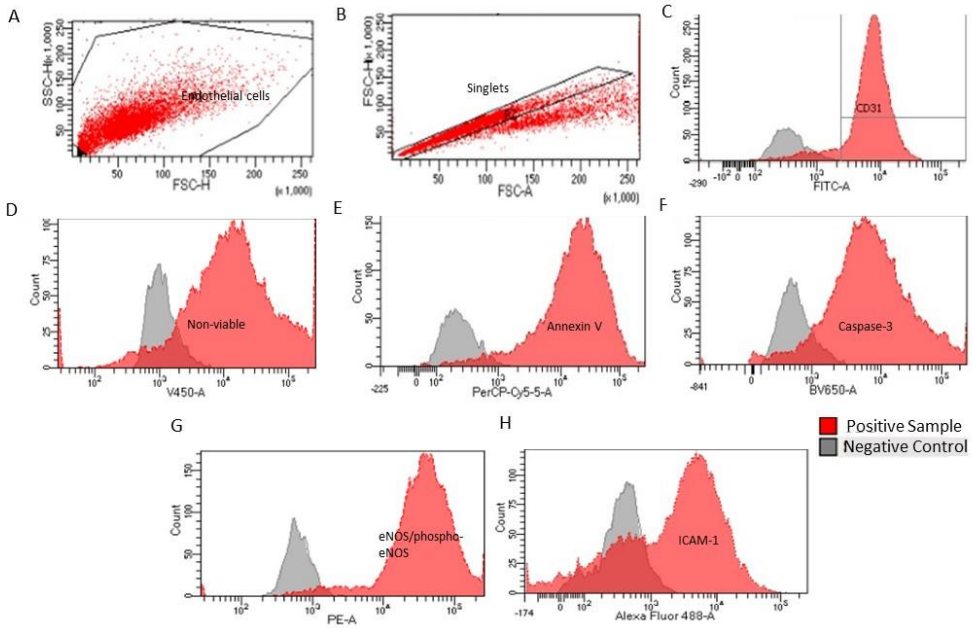

Figure 3.9. Representative gating strategy of endothelial cells by morphology (A), singlets (B), and CD31 expression as displayed by FITC histogram (C). Histograms of HUVEC cell viability (D), caspase-3 (E), Annexin-V (F), eNOS (G), and phospho-eNOS (H) expression are displayed as a function of their respective lasers (V450, PerCp-Cy.5, BV650, PE, respectively). *Histograms are displayed as a function of Mean Fluorescent Intensity.*

3.2.2.7 Flow Cytometry Compensation Strategy

Flow cytometry analysis using multiple fluorophores requires performance of fluorescent compensation to correct overlap of emission spectra from one channel into other active channels, as illustrated in **Figure 3.10**. The overlap is mathematically corrected via subtraction of spectral overlap in non-specific channels in order to mitigate false positive results (Mizrahi, Ish Shalom, Baniyash, & Klieger, 2018).

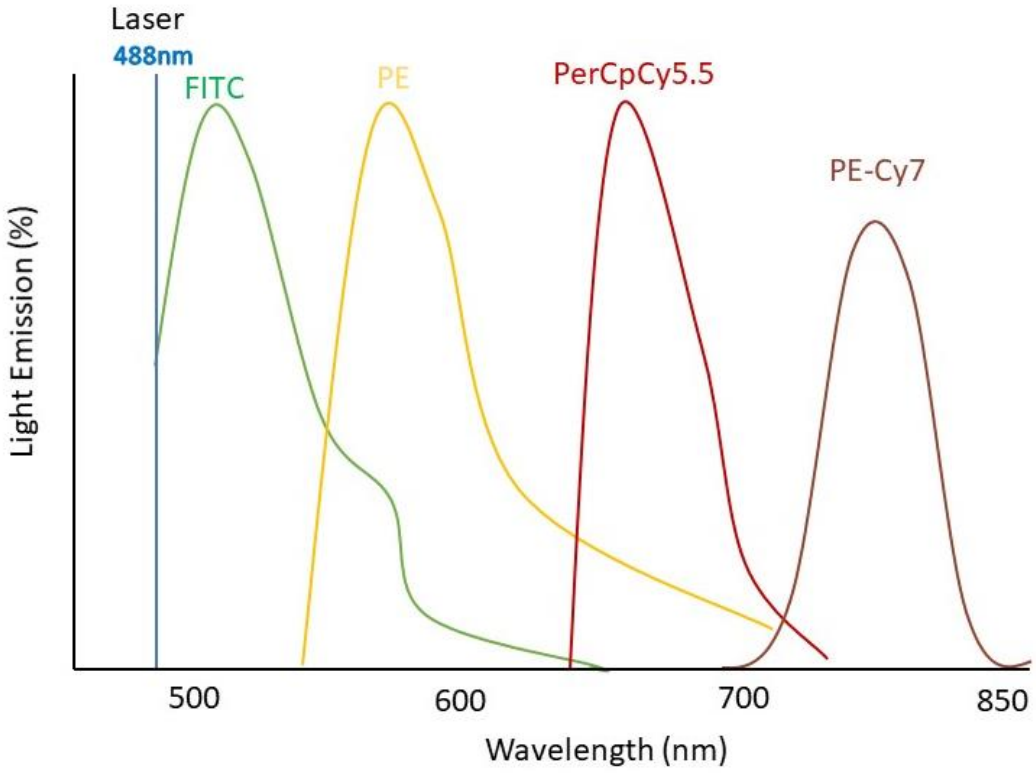

Figure 3.10. The overlap between emission spectra of fluorophores commonly used in flow cytometry.

Prior to initial data acquisition, compensation was performed to ensure electronic voltages were adjusted correctly to give quality visual plots and to avoid impingement of plots by high levels of compensation after data collection. Staining compensation beads with the individual antibodies used in the panel is the standard recommended protocol for compensation (Roederer, 2002). For the HUVEC studies, compensation was performed by incubating BD CompBeads with the same antibodies used in the panel and mathematically calculating fluorescence spill-over using FACSDiva 6.0 Software (BD Bioscience, USA).

Separate FACS tubes were used for each individual antibody included in the panel (anti-CD31-FITC, BioLegend, USA product code: 303104; viability dye-V450, BD Biosciences, USA, product code: 562247; anti-Annexin V-PerCP-Cy5.5, product code: 561431; BD Biosciences, USA; anti-cleaved caspase-3-BV650, BD Biosciences, USA, product code: 564096; anti-eNOS-PE, Miltenyi Biotec, Bergisch Gladbach, Germany, product code: 130-106-787; and anti-phospho-eNOS-PE Biorbyt, UK, product code: orb124607) and an unstained control was included. One drop of positive and one drop of negative BD CompBeads were added to 100µl of PBS. 1µl of corresponding antibody was added to the FACS tube solution and incubated for 30 minutes at 4^{o}C and protected from light. A wash step was performed by adding 1mL PBS to each FACS tube and centrifuged for 5 minutes at 400 x *g*. The supernatant was discarded, and beads were resuspended in 500µl of PBS. For acquisition, samples were run through 12-colour flow cytometer (FACS Celesta, BD Biosciences, USA) at a low flow rate. This method of compensation is illustrated for HUVEC panels in **Figure 3.11**. FACSDiva 6.0 Software (BD Bioscience, USA) was used to calculate the mathematical compensation required to eliminate fluorescent spill-over from fluorescence of each channel into the other active lasers in the panel. Once this was calculated, the mathematical correction was automatically applied to each experiment in the panel, ensuring data is accurate. The compensation values for HUVEC panels are presented in **Table 3.8 and 3.9**.

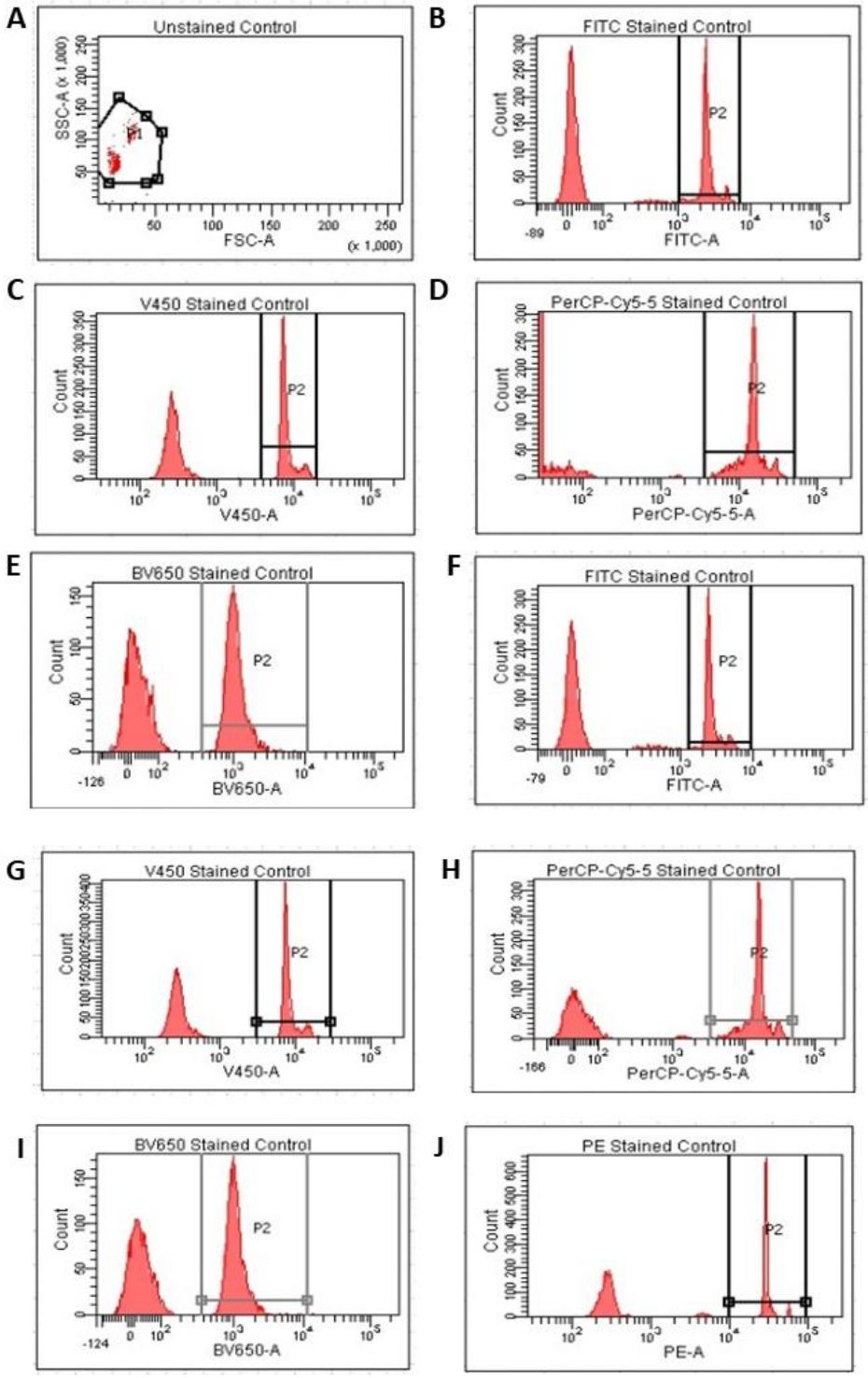

Figure 3.11. Compensation controls for HUVEC panels, with morphology plot (A) and stained controls for viability, Annexin V and caspase-3 panel (B-E) and for panels with additional eNOS and phospho-eNOS measurements (F-J), with positive controls for CD31-FITC (B, F), Viability-V450 (C, G), Annexin V (D, H), Caspase-3 (E, I), and eNOS/phospho-eNOS-PE (J).

Table 3.8 & 3.9. Compensation values for HUVEC panel 1 & 2.

Parameter	Parameter	Comp. (%)
BV650	V450	0.43
FITC	V450	0.18
PerCPCy5.5	V450	0.01
V450	BV650	16.17
FITC	BV650	1.6
PerCPCy5.5	BV650	7.46
V450	FITC	8.98
BV650	FITC	0.5
PerCPCy5.5	FITC	6.16
V450	PerCPCy5.5	0.05
BV650	PerCPCy5.5	11.86
FITC	PerCPCy5.5	0

Parameter	Parameter	Comp. (%)
BV650	V450	0.44
PerCPCy5.5	V450	0.01
PE	V450	0.09
FITC	V450	0.21
V450	BV650	13.33
PerCPCy5.5	BV650	7
PE	BV650	1.39
FITC	BV650	1.47
V450	PerCPCy5.5	0.04
BV650	PerCPCy5.5	11.87
PE	PerCPCy5.5	0.05
FITC	PerCPCy5.5	0
V450	PE	0.02
BV650	PE	1.75
PerCPCy5.5	PE	23.98
FITC	PE	0.6
V450	FITC	8.06
BV650	FITC	0.34
PerCPCy5.5	FITC	6.12
PE	FITC	1.04

For the HCAEC panels, the compensation beads could not be stained with the antibodies used in the panel as some of these were rat and rabbit antibodies (anti-CD31-FITC, BioLegend, USA, product code: 303104; anti-Annexin V-PerCP-Cy5.5, BD Biosciences, USA, product code: 56143; anti-cleaved caspase-3-V450, BD Biosciences, USA, product code: 560627; and anti-eNOS-PE, Miltenyi Biotec, Bergisch Gladbach, Germany, product code: 130-106-787). BD CompBeads were Set Anti-Mouse Ig, and therefore, only bind to mouse antibodies. It is possible to perform compensation with cells rather than beads, as long as the cells highly express the marker of interest to allow for a strong positive population to be detected. For the apoptosis panel, PBMCs were incubated with 10µM camptothecin for 24 hours to induce apoptosis. For acquisition, PBMCs were gated based on their FSC-SSC plot and lymphocytes were selected as the population of interest. CD31+ was detected with a clear positive population and apoptosis was successfully induced by camptothecin as a clear positive population was observed for anti-Annexin V-PerCPCy5.5 and anti-cleaved caspase-3-V450. However, PBMCs did not express eNOS and therefore, there was no positive population for the anti-eNOS-PE channel. Hence, compensation could not be performed in this manner. Since this alternative method of compensation failed, troubleshooting had to be progressed to a BD expert. Expert advice was that it is not absolutely essential to use the same antibody used in the panel: it simply needs to be on the same fluorophore. Therefore, it is possible to use any anti-mouse anti-V450 antibody to compensate for our anti-caspase-3-V450. Likewise, for all other mAbs. Therefore, this advice informed subsequent practice.

BD CompBeads (BD Bioscience, USA) were stained with the following singular antibodies: anti-CD31-FITC, BioLegend, USA product code: 303104; anti-Annexin V-PerCP-Cy5.5, BD Biosciences, USA, product code: 56143; anti-cleaved caspase-3-V450, BD Biosciences, USA, product code: 560627; and anti-eNOS-PE, Miltenyi Biotec, Bergisch Gladbach, Germany, product code: 130-106-787. Compensation was then calculated using the aforementioned method. The automated mathematical calculation was ~500% for PE into PerCPCy5.5. This is not an acceptable level of spill-over and therefore, further troubleshooting was required.

The compensation value is representative of how the voltages are set. If PE into PerCP-Cy5.5 is 500%, it can be assumed that the voltage for the PerCP-Cy5.5 is very high and/or the voltage for PE too low, creating some unbalanced settings. Therefore, the methods for

voltage setting had to be adjusted by using unstained HCAECs rather than setting voltages based on unstained compensation beads. Therefore, prior to sample acquisition, voltages were set using an unstained sample of HCAECs to determine the negative population for each fluorophore. Firstly, forward scatter and side scatter dot plots were created to determine the size and morphology of all events, allowing selection of the population of interest (HCAECs). A plot of forward scatter-area and -height was then created, gated on the population of interest. From this plot, singlets were selected. All subsequent plots were gated on singlets to remove any potential doublets from data analysis. Lastly, the voltages for the fluorescent parameters were adjusted to place the negative peaks slightly above channel 100. It is essential to perform this step using the same cells as used in data collection as these cells have their own autofluorescent properties which will be stronger than the autofluorescence of compensation beads. Once the voltages were set, compensation was then performed. Anti-Mouse Ig compensation beads (BD CompBeads Set, BD Biosciences, USA) were used alongside mouse-antibodies (anti-CD146-FITC, BD Pharmingen, USA, product code: 560846; anti-FoxP3-PE, BD Pharmingen, USA, product code, 560852; anti-CD4-V450, BD Horizon, USA, product code: 560345; anti-CD19-PerCPCy5.5, BD Pharmingen, USA, product code: 561295) which were not part of the flow cytometry panel, as before. Despite the voltages being set using the HCAECs, the unstained compensation beads are what must be recorded as the unstained-negative control for the compensation. This is an acceptable method for compensating as voltages are set according to the autofluoresence of the cells and the compensation calculated based on channel spill over. As voltages were set based on unstained HCAECs and then ran the compensation with the beads, the negative populations are not in the ideal place, but this is acceptable as there is more autoflouresence with the cells. Positive samples (stained compbeads) were then recorded, and compensation calculated by FACSDiva software, as before. The compensation values for HCAEC panel 1 are presented in **Table 3.10.**

The same method was used for HCAEC CAM panel (anti-CD31-FITC, BioLegend, USA product code: 303104; anti-ICAM-1 CD54-AlexaFluor 488, BioLegend, UK, product code: 322713) as antibodies were also rat, not mouse. Therefore, voltages were set based on the negative population of HCAECs. CompBeads were stained singularly with anti-VEGF-AlexaFluor488 mAb (R&D Systems, USA, product code: IC2931G). The compensation values for HCAEC

panel 2 are presented in **Table 3.11.** All compensation plots for HCAEC panels are illustrated in **Figure 3.11**.

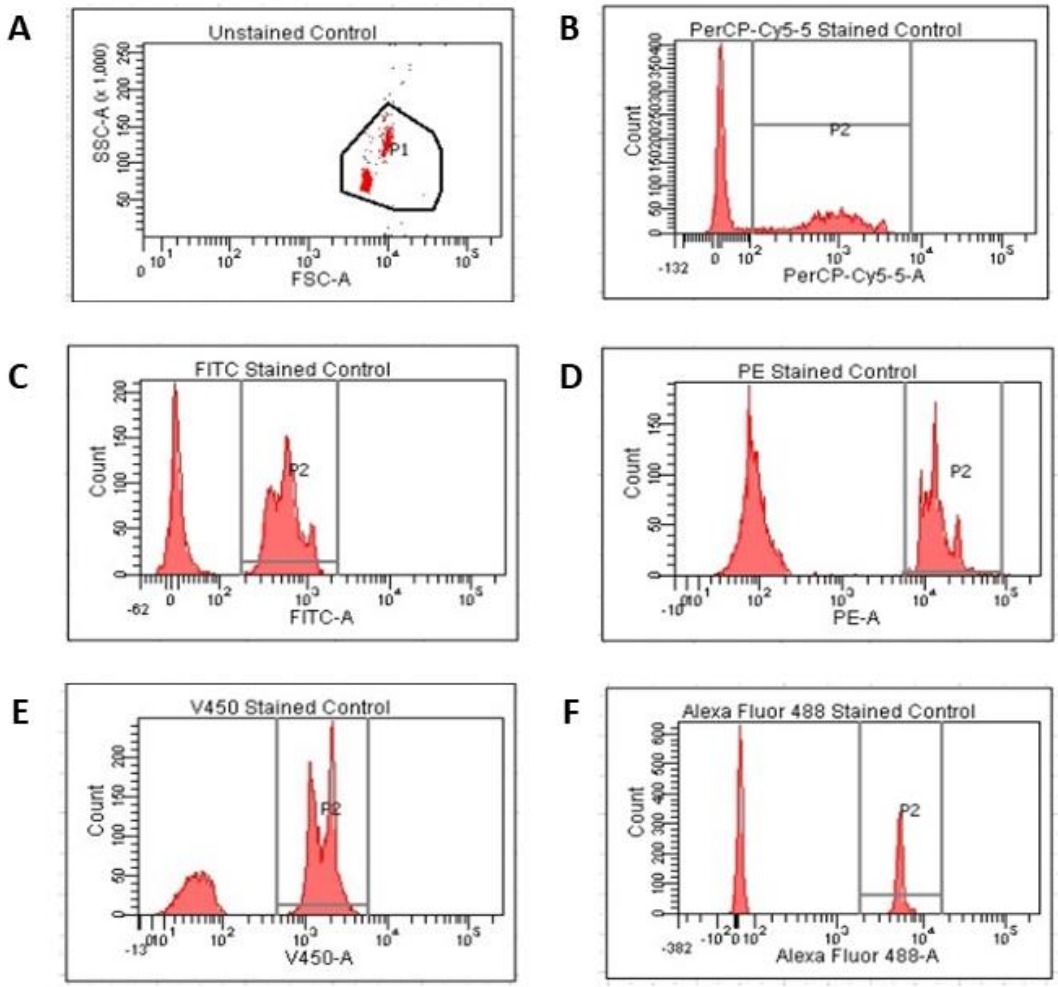

Figure 3.12. Compensation controls for HCAEC panels, with morphology plot (A) and stained controls for CD31-FITC (B), Annexin V-PerCpCy5.5 (C), Caspase-3-V450 (D), eNOS-PE (E), and ICAM-1/CD54-AlexaFluor488 (F).

Table 3.10 & 3.11. Compensation values for HCAEC panel 1 &2.

Parameter	Parameter	Comp (%)
PE	PerCP-Cy5-5	3.82
FITC	PerCP-Cy5-5	0.12
V450	PerCP-Cy5-5	2.08
PerCP-Cy5-5	PE	13.73
FITC	PE	1.13
V450	PE	0.08
PerCP-Cy5-5	FITC	1.37
PE	FITC	2.88
V450	FITC	2.75
PerCP-Cy5-5	V450	0.12
PE	V450	3.20
FITC	V450	0.52

Parameter	Parameter	Comp (%)
Alex Fluor 488	FITC	0.22
FITC	Alexa Fluor 488	0.72

3.2.2.8 Data Analysis of Flow Cytometry Experiments

Data were firstly analysed using FACSDiva 6.0 Software (BD Bioscience, USA) to create dot plots and histograms which could be gated to generate the numerical data. FACSDiva 6.0 Software (BD Bioscience, USA) was then used to further analyse the data was and histograms were overlayed to create a visual representation of the effects of cell conditions on antibody expression. Experiments were performed in biological triplicate and pooled, allowing statistical analysis to be performed.

3.2.3 Endothelial Cell Wound Healing Assay

A cell wound healing assay is an economical method to study cell migration *in vitro*. This method is based on the observation that, upon mechanical infliction of a wound on a confluent cell monolayer, the cells on the edge of the wound will migrate/proliferate to 'heal' the wound (Grada, Otero-Vinas, Prieto-Castrillo, Obagi, & Falanga, 2017b). This technique has been effectively utilised in several cell types, including epithelial, corneal, renal, intestinal, and the endothelium (Vidmar, Chingwaru, & Chingwaru, 2017), and has contributed to knowledge of cell behaviour in several research areas, including oncology (R. Sullivan et al., 2011), diabetes (Tam et al., 2011), and immunology (Vidmar et al., 2017). For endothelial cells in particular, analysis of this process is important as the ability of the endothelium to repair itself *in vivo* is critical for maintaining the integrity of the vasculature and is involved in preventing the initiation of atherosclerosis (Cooke & Tsao, 2011; Insull, 2009).

Endothelial cells were cultured in collagen coated 24-well plates and treated with medium containing 5% human serum (for specific details of the human serum, see detail in Study *Chapters 5, 6 and 7*) and incubated at 37^{o}C 5% CO_2, as outlined above. After 24-hours of serum preconditioning, the endothelial monolayer was scratched using 200µl pipette. The monolayer was then washed twice with 200µl PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002) to remove debris. Immediately after mechanical injury was inflicted, endothelial cells were exposed to physiological concentrations of FEC-T drugs and re-incubated at 37^{o}C 5% CO_2. Drugs were washed off with PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002) at corresponding time points (as above) and replaced with fresh ECGM (Sigma-Aldrich, Gillingham, UK, product code: 211-500).

Cells were imaged using Primovert Axiocam ERc5s microscope (ZEISS, Germany) using x4 magnification at 0 hours and then subsequent corresponding time points (as above) plus 24 -hour time-point. This process is illustrated in **Figure 3.13**.

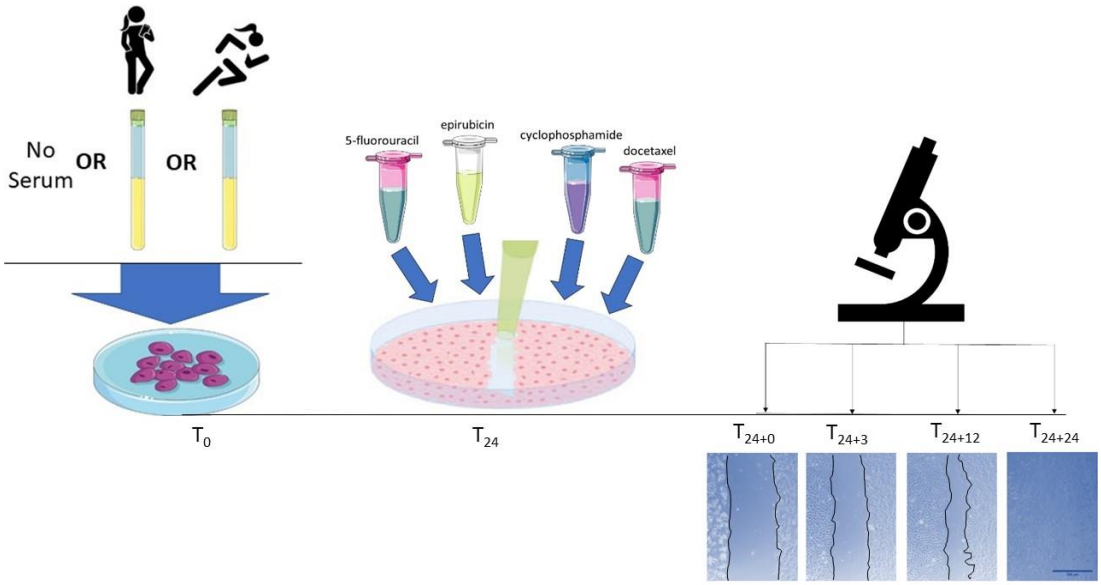

Figure 3.13. Schematic protocol for HUVEC serum preconditioning, wound induction, FEC-T exposure, and microscopy. *Scale bar is 500µm.*

Rate of gap closure was analysed using image analysis software (Image J 1.x, Java, USA). After measuring the gap area for each image, gap area was plotted as a function of time to derive the rate of wound closure. Note that proliferation was not inhibited in this assay and therefore, wound closure is likely a function of cellular migration-proliferation, and not migration only (Grada, Otero-Vinas, Prieto-Castrillo, Obagi, & Falanga, 2017a). Hence, the rate of wound closure is defined as the average velocity at which the cells collectively move into the gap. For analyses where the rate of wound closure was not different between groups, the $t_{1/2gap}$ value (the point at which the gap is half the original area) was calculated. Calculations for rate of wound closure and $t_{1/2gap}$ are as follows (Jonkman et al., 2014):

$$t1/2gap = \frac{InitialGapArea}{2 \times |slope|}$$

$$Vmigration = \frac{|slope|}{2 \times l}$$

Area under the curve (AUC) was also calculated to create a visual representation of the change in the gap size over time.

The overall in vitro experimental protocol, including flow cytometry and wound healing analysis techniques is outlined in **Figure 3.14**.

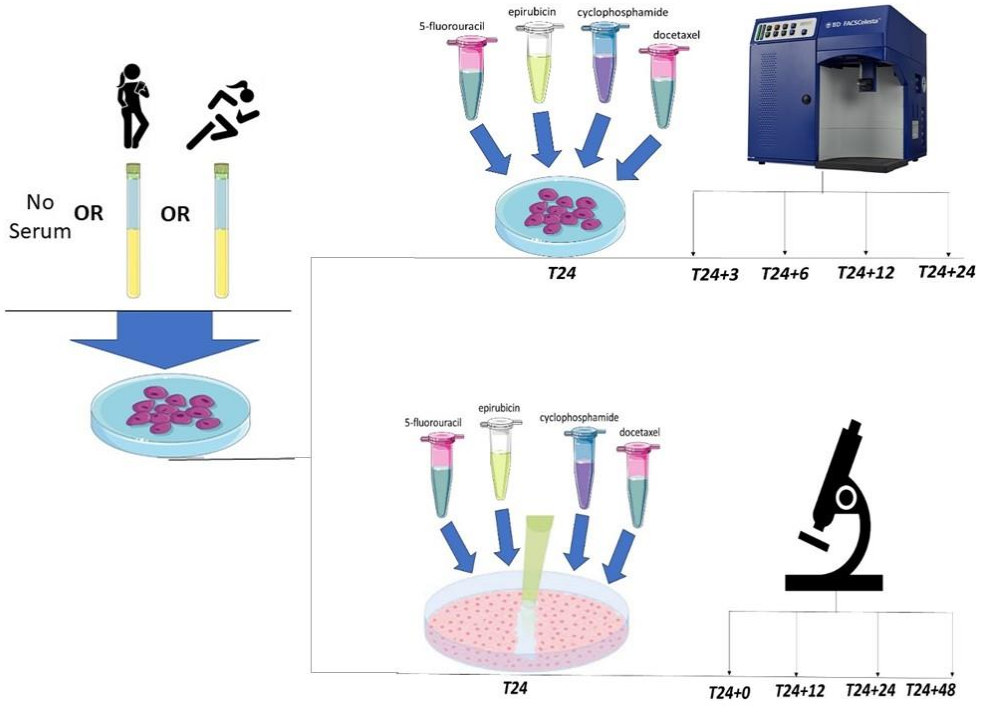

Figure 3.14. Schematic of experimental protocol with 24-hours serological preconditioning of endothelial cells before chemotherapy exposure, analysed by flow cytometry and wound healing assay.

3.3 Statistical Analysis

Shapiro-Wilks test of normality were performed using GraphPad Prism Version 9.0.1 (USA). All data was determined to be normally distributed. Several one-way analyses of variance (ANOVA) were performed to determine the effect of different drugs (5-FU, epirubicin, cyclophosphamide and docetaxel, separately and FEC combination) and concentrations (control [0], low dose, physiological dose, high dose) on endothelial cell viability and apoptosis drug conditions to the no drug control.

Additionally, One-way ANOVAs were performed to determine differences between DMSO vehicle control, chemotherapy without serum conditioning (results taken from *Chapter 4*) and both serum conditions (active and inactive) on endothelial cell viability, apoptosis,

eNOS, ICAM-1 expression and wound healing (AUC, $V_{migration}$, $T_{1/2\ gap}$) for each of the drug conditions (5-FU, epirubicin, cyclophosphamide and docetaxel, separately and FEC combination); and for each of the serum conditions. Statistical significance was assumed if $p < 0.05$. Specific statistical analyses are detailed in relevant study *Chapters 4, 5, 6, and 7*.

Chapter 4

The toxic effects of FEC-T chemotherapy exposure on human vascular endothelial cells

4.1 Introduction

The toxicity of chemotherapy treatments for BC is attributed to CVD being the leading cause of mortality in BC survivors, responsible for ~16% of deaths (Patnaik, Byers, DiGuiseppi, Dabelea, & Denberg, 2011b). So far, research regarding chemotherapy-induced toxicity has focused mainly on the cardiac mechanisms of toxicity. However, it is important to also consider the role of the vasculature as this is the first point of contact with chemotherapy drugs and is also damaged with chemotherapy exposure, as shown by increased CECs (Beerepoot et al., 2004) and endothelial dysfunction determined by reduced FMD in BC patients after receiving chemotherapy (Duquaine et al., 2003b). Endothelial damage and dysfunction often precedes cardiac toxicity with chemotherapy (Ching et al., 2021; Verma et al., 2003), and is associated with atherosclerosis development (Winther et al., 2016) and cardiac events, including MI and sudden cardiac death (Cameron et al., 2016a). Despite the clear importance of the endothelium for cardiovascular health and its potential role in chemotherapy-induced toxicity, there is not yet sufficient evidence for the role of endothelial cell viability and cellular function to explain the toxic vascular side effects of chemotherapy treatment for BC. determining the role of the vascular endothelium in chemotherapy toxicity is of interest to determine if there is potential for targeted therapies or early interventions to prevent or manage toxicity.

This study aims to investigate the effects of commonly used BC chemotherapy treatments on endothelial cell health using an *in vitro* model of endothelial cell toxicity (*aim 1*). To achieve *aim 1*, *in vitro* models of chemotherapy-induced toxicity were developed using human umbilical vein endothelial cells (HUVEC) and human coronary artery endothelial cells (HCAEC). A variety of measures of endothelial health were used as outcomes measures, including cell viability, apoptosis, caspase-3 activation, eNOS content and adhesion molecule expression.

It is hypothesised that chemotherapy will reduce cell viability, increase cell apoptosis and adhesion molecule expression, and reduce NO production/activation in a dose- and time-dependent manner.

4.2 Methodology

4.2.1 Endothelial Cell Culture

Unpooled human umbilical vascular endothelial cells (HUVECs) (product code: C0035C, Thermofisher Scientific, Waltham, USA) and human coronary artery endothelial cells (HCAECs) (product code: 300-05A, Sigma-Aldrich, Gillingham, UK) were cultured in endothelial cell growth medium (ECGM; product code: 211-500, Sigma-Aldrich, Gillingham, UK) and Meso-endothelial cell growth medium (MEGM; product code: 212-500, Sigma-Aldrich, Gillingham, UK), respectively, and incubated at 37^{o}C in 5% CO_2 for 24 hours.

4.2.2 Endothelial Cell Chemotherapy Exposure

Appropriate concentrations of 5-fluorouracil (product code: ab142387; Abcam, UK), epirubicin hydrochloride (product code: ab142100; Abcam, UK), cyclophosphamide (product code: ab141240, Abcam, UK) and docetaxel (product code: ab141248; Abcam, UK) were added to the appropriate corresponding wells. In HUVECs this was performed first as single drugs and then in combination (FEC), as commonly prescribed in early-stage BC treatment. In HCAECs study, FEC combined and docetaxel treatments were used only, as this is the common chemotherapy regimen used to treat breast cancer (Roché et al., 2006). Drug concentrations were based on the serum concentration found in patients after exposure to each of these drugs (5-FU: 1.5µM (Reigner et al., 2003); epirubicin: 0.006µM (Danesi et al., 2002); cyclophosphamide: 38µM (Adams et al., 2014); and docetaxel: 6µM (Hurria et al., 2006)). A concentration 50% above and 50% below the physiological level was included to allow a potential dose-response effect to be investigated, and an additional well with media only was used as a no drug control. Drugs were washed off at time-points corresponding to the literature findings when serum levels of each drug were diminished (Adams et al., 2014; Danesi et al., 2002; Hurria et al., 2006; Reigner et al., 2003), and re-incubated at 37^{o}C in 5% CO_2 until the appropriate analysis time-points, whereby the literature reports the most potent effects of the drugs. These time-points are as follows: 5-FU: 3 and 12 hours; epirubicin: 4 and 12 hours; cyclophosphamide: 3 and 6 hours (Chow & Loo, 2003); and docetaxel: 24 and 48 hours (Morse et al., 2005); and for FEC: 4 and 12 hours.

4.2.3. Flow Cytometry Methodology

4.2.3.1 Sample Preparation for Analysis of Drug Effects

At the respective time-points, media was removed, cells were washed with 200µl NaCl, and 100µl trypsin-EDTA (Gibco, Thermofisher Scientific, Waltham, USA) solution was used for cell detachment. Cells were transferred to a 96 v-bottom plate and centrifuged at 300 x *g* for 3 minutes at 21°C. Supernatant was discarded and cells resuspended in 100µl PBS (product code: 003002, Invitrogen, Thermofisher, Waltham, USA) for analysis by flow cytometry.

4.2.3.2 Flow Cytometry

The effects of chemotherapy on HUVEC and HCAEC viability, apoptosis and eNOS content were determined by flow cytometry using a panel of mAbs. The optimal working antibody concentrations were determined by titration experiments using whole blood samples. 100,000 HUVECs were stained with anti-CD31-FITC (endothelial cell marker; product code: 303104; BioLegend, USA), fixable viability stain-V450 (product code: 562247; BD Biosciences, USA), and anti-Annexin V-PerCP-Cy5.5 (product code: 561431; BD Biosciences, USA) and incubated for 30 minutes at 4°C. HCAECs were stained with anti-CD31-FITC (endothelial cell marker; product code: 303104; BioLegend, USA) and anti-Annexin V-PerCP-Cy5.5 (product code: 561431; BD Biosciences, USA) incubated for 30 minutes at 4°C. Cells were then permeabilised for intracellular staining, using 100µl fixation and permeabilization solution (product code: 554714; BD Biosciences, USA) and incubated for 20 minutes at 4°C. The cells were washed twice with 100µl perm wash buffer (product code: 554714; BD Biosciences, USA). HUVECs were then incubated with anti-cleaved caspase-3-BV650 (product code: 564096; BD Biosciences, USA) for 30 minutes at 4°C. For FEC combined treatment only, HUVECs were additionally stained with anti-eNOS-PE (product code: 130-106-787; Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-phospho-eNOS-PE (product code: orb124607; Biorbyt, UK) mAbs were also added (to separate wells as on same fluorophore). HCAECs were intracellularly stained with anti-cleaved caspase-3-V450 (product code: 560627; BD Biosciences, USA) and anti-eNOS-PE (product code: 130-106-787; Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were incubated for a further 30 minutes at 4°C, washed twice with 100µl perm wash buffer (product code: 554714; BD Biosciences,

USA) and resuspended in 200µl PBS (product code: 003002, Invitrogen, Thermofisher, Waltham, USA).

For HCAEC experiments, an additional panel was used to determine adhesion molecule expression. HCAECs were fixed and stained with antibodies in-plate before detachment. HCAEC monolayers were fixed with 100µl 0.5% formaldehyde at 4°C for 5 minutes, washed with 100µl PBS and re-incubated in 100µl PBS (0.1% bovine serum albumin) with anti-ICAM-1 CD54-AlexaFluor 488 (product code: 322713, BioLegend, UK) for 30 min at 4°C. The HCAEC monolayer was washed with 100µl PBS before detachment with 100µl trypsin-EDTA (Gibco, Thermofisher Scientific, Waltham, USA) solution. Cells were transferred to a 96 v-bottom plate and centrifuged at 300 x *g* for 3 minutes at 21°C. Supernatant was discarded and cells resuspended in 200µl PBS (product code: 003002, Invitrogen, Thermofisher, Waltham, USA) for analysis by flow cytometry.

All samples were analysed using High Throughput System (HTS) on a 12-colour flow cytometer (FACS Celesta, BD Biosciences, USA). Flow cytometer setup and calibration were performed using CS&T beads (BD Biosciences, USA). For sample acquisition, the cells were set as stopping gate of 10000 events. Data were acquired using FACSDiva 6.0 Software (BD Bioscience, USA). Fluorescence compensation was performed using compensation beads (BD CompBeads Set, BD Biosciences, USA).

4.2.3.3 Flow Cytometry Gating Process

Flow cytometer setup and calibration were performed using CS&T beads (BD Biosciences, USA). For sample acquisition, a minimum of 10,000 cell events were collected. Data were acquired using FACSDiva 6.0 Software (BD Bioscience, USA). Fluorescence compensation was performed using compensation beads (BD CompBeads Set, BD Biosciences, USA).

Firstly, forward scatter and side scatter dot plots were created to determine the size and morphology of all events, allowing selection of the population of interest. A plot of forward scatter-area and -height was then created, gated on the population of interest. From this plot, singlets were selected. All subsequent plots were gated on singlets to remove any potential doublets from data analysis. FITC-CD31$^+$ expression confirmed endothelial cell identity. Once gated on CD31$^+$ cells, dot plots and histograms of viability-V450, annexin V-PerCP-Cy, eNOS-PE, and phospho-eNOS-PE could be visualised and data interpreted for

HUVECs. For HCAECs, dot plots and histograms of cleaved caspase-3-V450, annexin V-PerCP-Cy5.5 and eNOS-PE were used. Additionally, for HCAECs, ICAM-AlexaFluor 488 was analysed in the same way using a separate panel. Representative gating strategies are shown in **Figure 4.1**.

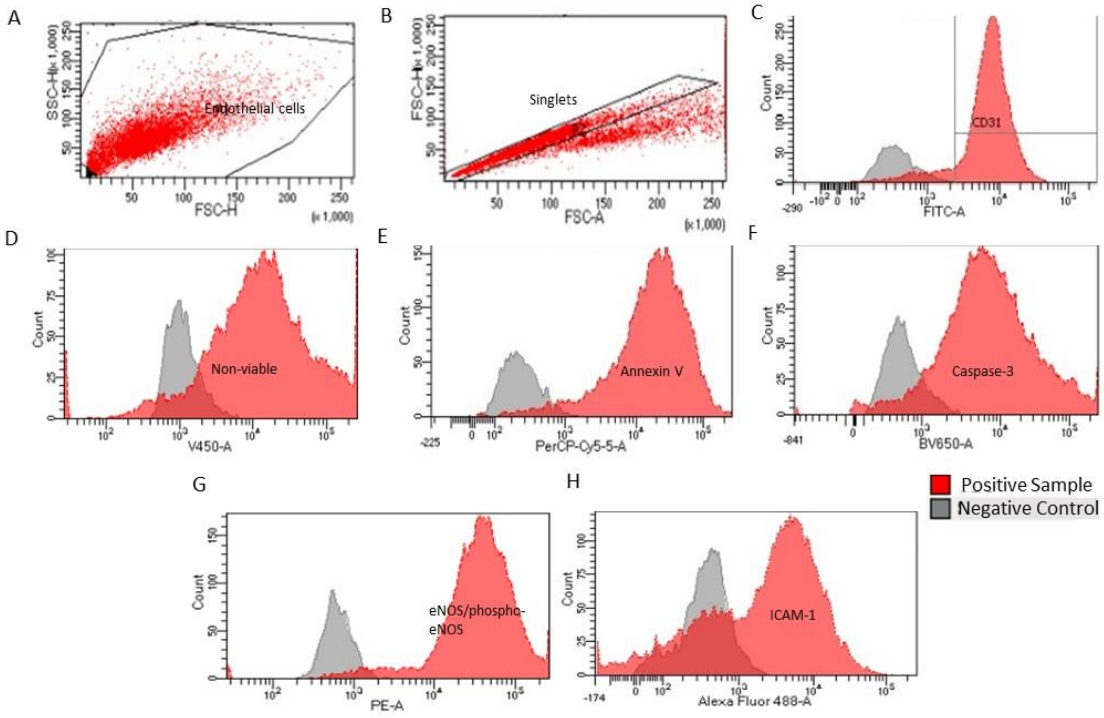

Figure 4.1. Representative gating strategy of endothelial cells by morphology (A), singlets (B), and CD31 expression as displayed by FITC histogram (C). Histograms of cell viability (D), cleaved caspase-3 (E), Annexin-V (F), eNOS/phospho-eNOS (G), and ICAM-1 (H) expression are displayed as a function of their respective lasers (V450, PerCp-Cy.5, BV650, PE, and AlexaFluor 488, respectively). *Histograms are displayed as GeoMean (Mean Fluorescent Intensity (MFI)).*

4.2.4 Wound Healing Assay

Endothelial cells were cultured in 24 well plates and treated with medium containing 5% habitually active or sedentary serum and incubated at 37°C 5% CO_2, as outlined above. After 24-hours of serum preconditioning, the endothelial monolayer was scratched using a 200µl pipette, and washed to remove debris. Immediately after mechanical injury was inflicted, endothelial cells were exposed to physiological concentrations of FEC-T drugs and re-incubated at 37°C 5% CO_2. Drugs were washed off at corresponding time points and replaced with fresh media. Cells were imaged using Primovert Axiocam ERc5s microscope

(ZEISS, Germany) using x4 magnification at 0, 3-, 4-, 6-, 12-, 24- and 48-hours. Rate of gap closure was analysed using image analysis software (Image J 1.x, Java, USA). After measuring the gap area for each image, gap area was plotted as a function of time to derive the rate of wound closure. For analyses where the rate of wound closure was not different between groups, the $t_{1/2gap}$ value (the point at which the gap is half the original area) was also calculated. Calculations for rate of wound closure and $t_{1/2gap}$ are as follows (Jonkman et al., 2014):

$$\mathrm{t1/2gap} = \frac{\mathrm{InitialGapArea}}{2 \times |\mathrm{slope}|}$$

$$\mathrm{Vmigration} = \frac{|\mathrm{slope}|}{2 \times \mathrm{l}}$$

AUC was also calculated to create a visual representation of the change in the gap size over time.

4.2.5 Statistical Analysis

All experiments were performed in biological triplicate and pooled, allowing statistical analysis to be performed. Shapiro-Wilks test of normality were performed. All data was determined to be normally distributed. Several repeated measures one-way analyses of variance (ANOVA) were performed using GraphPad Prism Version 9.0.1 (USA) to determine the effect of different drugs (5-FU, epirubicin, cyclophosphamide and docetaxel, separately and FEC combination) and concentrations (media only, DMSO control, low dose, physiological dose, and high dose) on endothelial cell viability, apoptosis, eNOS, ICAM-1, and wound healing (AUC, $V_{migration}$ and $T_{1/2gap}$), comparing drug conditions to the DMSO (no drug) vehicle control.

4.3 Results

4.3.1 Dose Response Effects of FEC-T Drugs on Endothelial Cell Morphology

From cell images, it can be observed that control cells (**Figure 4.2**) are live and growing with the expected morphology. As drugs are added, there is a reduced number of adherent cells with a morphology which has changed to more shrunken cells which tend to clump together in both HUVECs and HCAECs (**Figure 4.2**).

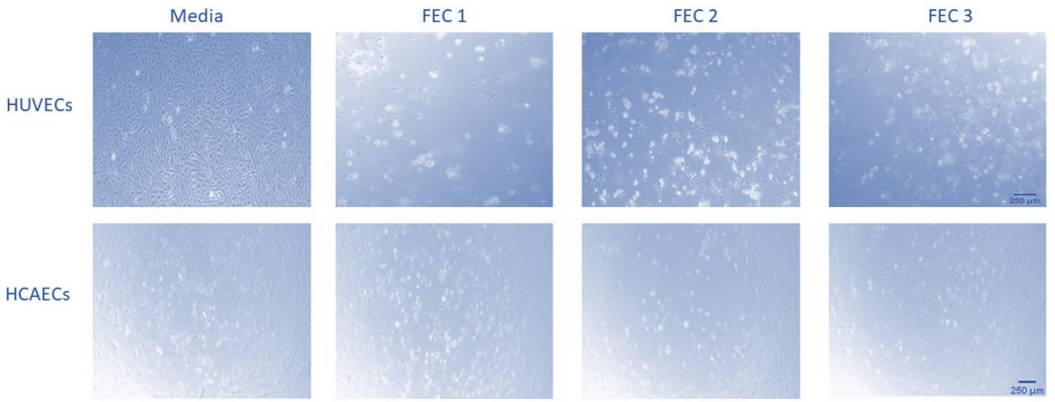

Figure 4.2. Representative images of passage 5 HUVECs and passage 4 HCAECs at 4 hours post-exposure to media only control, FEC 1, FEC 2, and FEC 3 drugs conditions. *Images are X4 magnification. Scale bars are 250µm. (FEC 1 = 5-FU: 1 µM, epirubicin: 0.003µM, cyclophosphamide: 19µM. FEC 2 = 5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM. FEC 3 = 5-FU: 2µM, epirubicin: 0.009µM, cyclophosphamide: 57µM).*

Results from HUVEC studies influenced the protocols for subsequent HCAEC studies. Drug treatments chosen were FEC combined and docetaxel alone, as commonly prescribed in early-breast cancer care (Roché et al., 2006). One time-point was chosen for analysis based on the most potent time-point from the HUVEC study. For FEC there was no effect of time on drug potency and therefore, the 4-hour time point was chosen for FEC experiments. For docetaxel, the 48-hour time-point was most potent to the HUVECs and hence this time-point was selected for subsequent HCAEC studies.

4.3.2 Dose Response Effects of FEC-T Drugs on Endothelial Cell Viability, Annexin-V and Cleaved Caspase-3 Expression

One-way ANOVA analysis between 5-FU drug conditions identified several significant results. Fluorouracil significantly reduced HUVEC viability at 2µM (**Figure 4.3A&D**). Early apoptosis, as measured by Annexin V binding to phosphatidylserine, significantly increased at both 3 hours (**Figure 4.3B**) and 12 hours post-exposure (**Figure 4.3E**) in a dose-dependent manner. In addition, 5-FU elevated cleaved caspase-3 expression 3-hours post-exposure (**Figure 4.3C**).

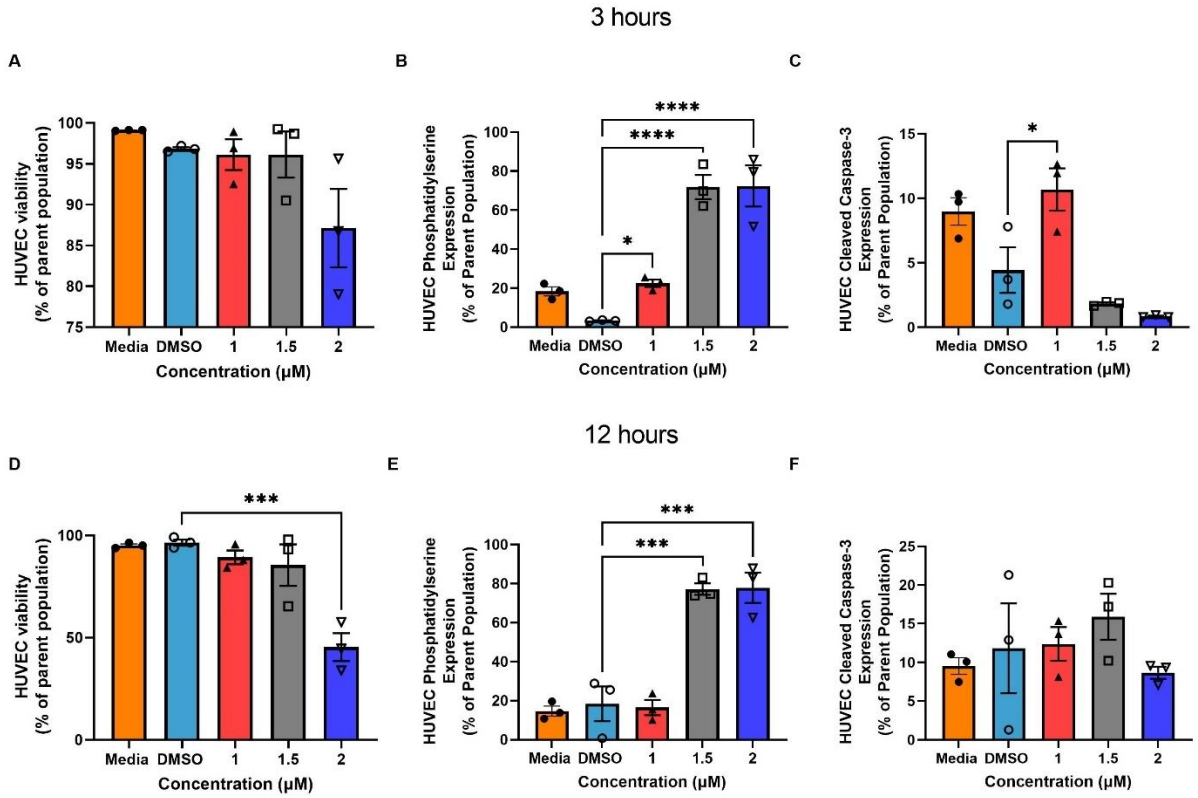

Figure 4.3. Dose-response effects of 5-fluorouracil on HUVEC cell viability (A, D), phosphatidylserine (B, E) and cleaved caspase-3 (C, F) expression, across a range of physiological concentrations at 3- (A, B, C) and 12-hours (D, E, F), as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001*.

Epirubicin also significantly reduced HUVEC viability in a dose-dependent manner at 4-hours post-exposure (**Figure 4.4A**) but was not evident at 12-hours (**Figure 4.4D**). This occurred alongside dose-dependently elevated cleaved caspase-3 and phosphatidylserine expression at both 4- and 12-hours (**Figure 4.4B, C, E and F**).

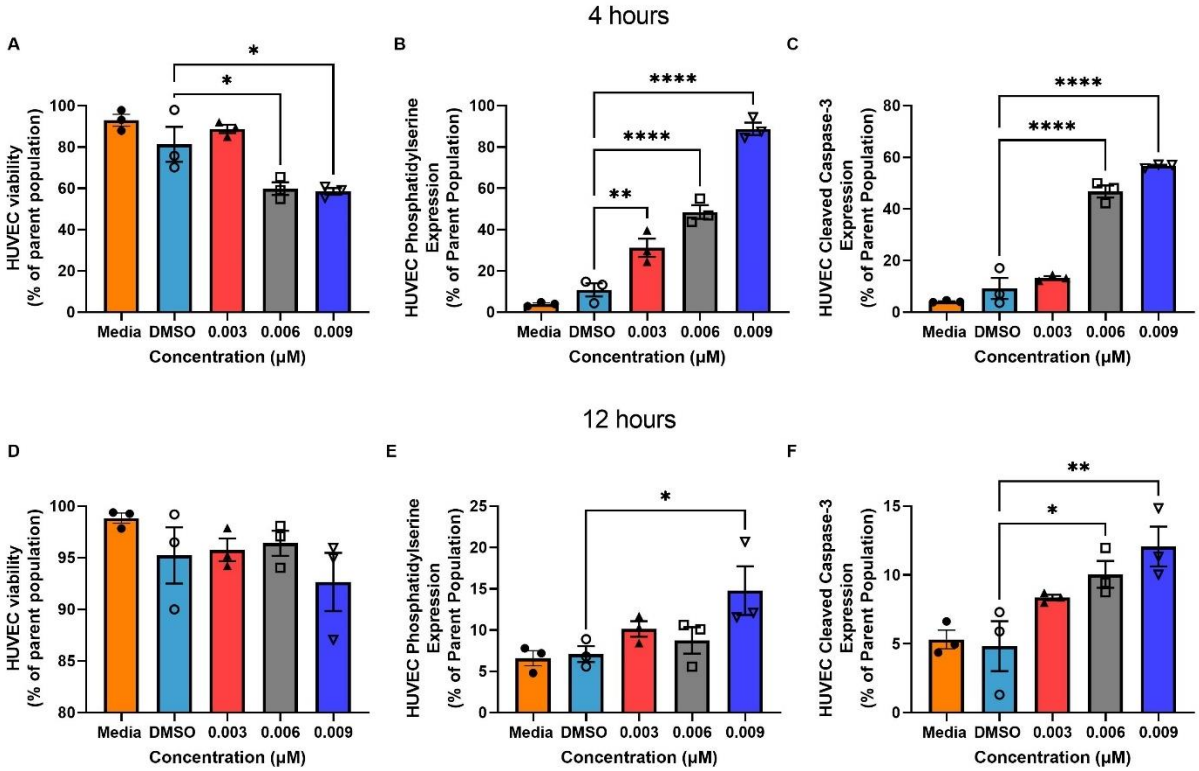

Figure 4.4. Dose-response effects of epirubicin on HUVEC cell viability (A, D), phosphatidylserine (B, E) and cleaved caspase-3 expression (C, F), across a range of physiological concentrations at 4- (A, B, C) and 12-hours (D, E, F), as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001.*

Similar trends are observed with cyclophosphamide exposure, with significantly reduced HUVEC viability in a dose-dependent manner at both 3- and 6 hours (**Figure 4.5A and D**). phosphatidylserine expression increased significantly at 3 hours (**Figure 4.5B**) post-exposure with moderate and high dosages of cyclophosphamide but phosphatidylserine expression (Annexin V binding) with the lower 19µM dosage did not reach significance. However, at 6-hours, phosphatidylserine elevation did not reach significance for any of the cyclophosphamide conditions (**Figure 4.5E**). There was also significantly elevated cleaved caspase-3 expression with the 38µM and 57µM cyclophosphamide at 3 hours (**Figure 4.5C**), with only non-significantly elevated expression at 6 hours (**Figure 4.5F**).

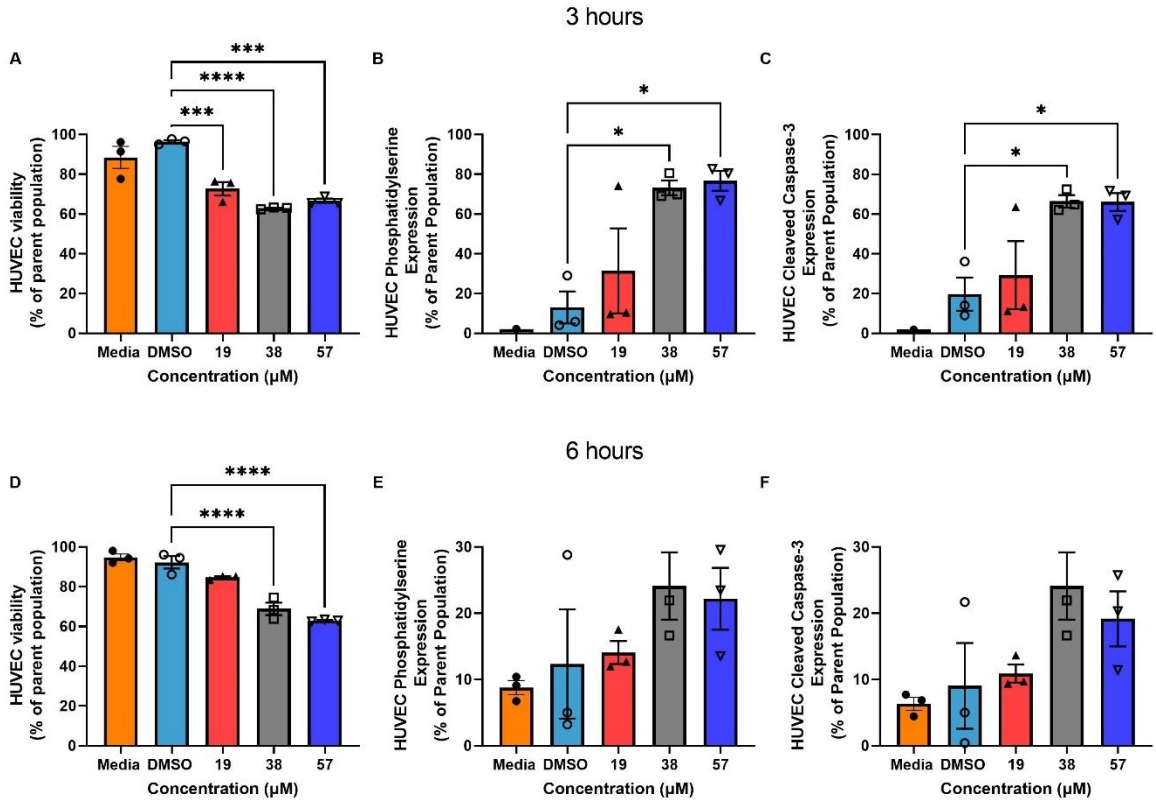

Figure 4.5. Dose-response effects of cyclophosphamide on HUVEC cell viability (A, D), phosphatidylserine (B, E) and cleaved caspase-3 expression (C, F), across a range of physiological concentrations at 3- (A, B, C) and 6-hours (D, E, F), as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001.*

FEC combined treatment also had several significant differences revealed by one-way ANOVA analysis (**Figure 4.6**). For ease of interpretation, drug combinations are displayed as 'FEC 1', 'FEC 2' and 'FEC 3'. Full details of drug concentrations used are displayed in **Table 4.1**. FEC significantly reduced HUVEC viability in a dose-dependent manner at 4- and 12-hours post-exposure (**Figure 4.6A & 6D)**. At 4-hours and 12-hours, HUVEC phosphatidylserine (Annexin V binding) and cleaved caspase-3 expression increased in a dose-dependent manner **(Figure 4.6B, C, E, F)**. Similarly, in HCAECs both markers of early- and late-apoptosis increased in a dose-dependent manner **(Figure 4.7)**.

Table 4.1. Range of physiological concentrations used for FEC combined exposure.

FEC 1	**FEC 2**	**FEC 3**
5-FU: 1 µM epirubicin: 0.003µM cyclophosphamide: 19µM	5-FU: 1.5 µM epirubicin: 0.006µM cyclophosphamide: 38µM.	5-FU: 2µM epirubicin: 0.009µM cyclophosphamide: 57µM

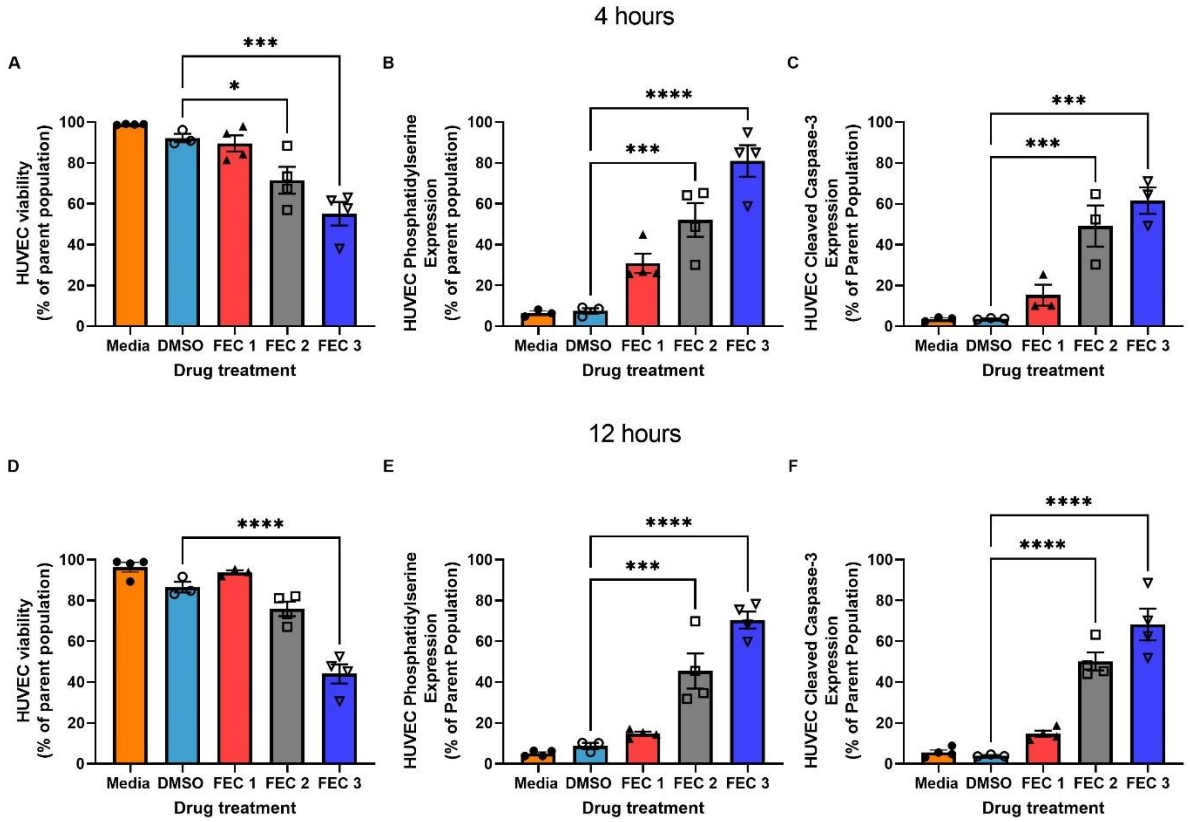

Figure 4.6. Dose-response effects of combined 5-fluorourcil, epirubicin and cyclophosphamide (FEC) on HUVEC cell viability (A, D), phosphatidylserine (B, E) and cleaved caspase-3 (C, F) expression at 4- (A, B, C) and 12-hours (D, E, F) across a range of physiological concentrations (FEC 1, FEC 2, and FEC 3), as determined by ANOVA. *Data shown are mean ± SEM (n=5). *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001.*

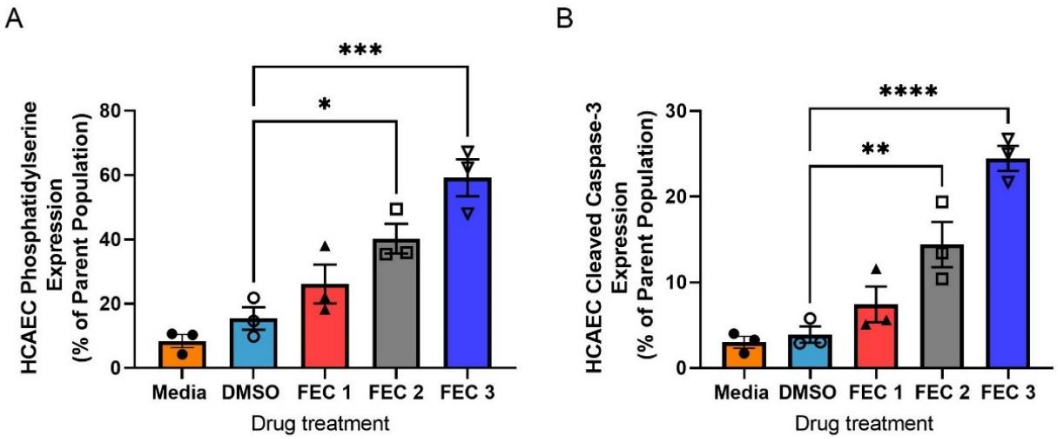

Figure 4.7. Dose-response effects of combined 5-fluorourcil, epirubicin and cyclophosphamide (FEC) on HCAEC cell viability (A), phosphatidylserine (B) and cleaved caspase-3 (C) expression at 4-hours across a range of physiological concentrations (FEC 1, FEC 2, and FEC 3), as determined by ANOVA. *Data shown are mean ± SEM (n=3). ** p < 0.01, ****p < 0.0001.*

More significant effects have been found with HUVEC exposure to docetaxel (**Figure 4.8**), which significantly reduced cell viability in a time-dependent manner across all dosages (**Figure 4.8A and D**). This was accompanied by a significantly elevated Annexin V binding to phosphatidylserine and cleaved caspase-3 expression at both 24- and 48-hours post-exposure (**Figure 4.8B, C**, **E and F**). In HCAECs, both Annexin V and cleaved caspase-3 expression increased with physiological concentrations of docetaxel **(Figure 4.9)**.

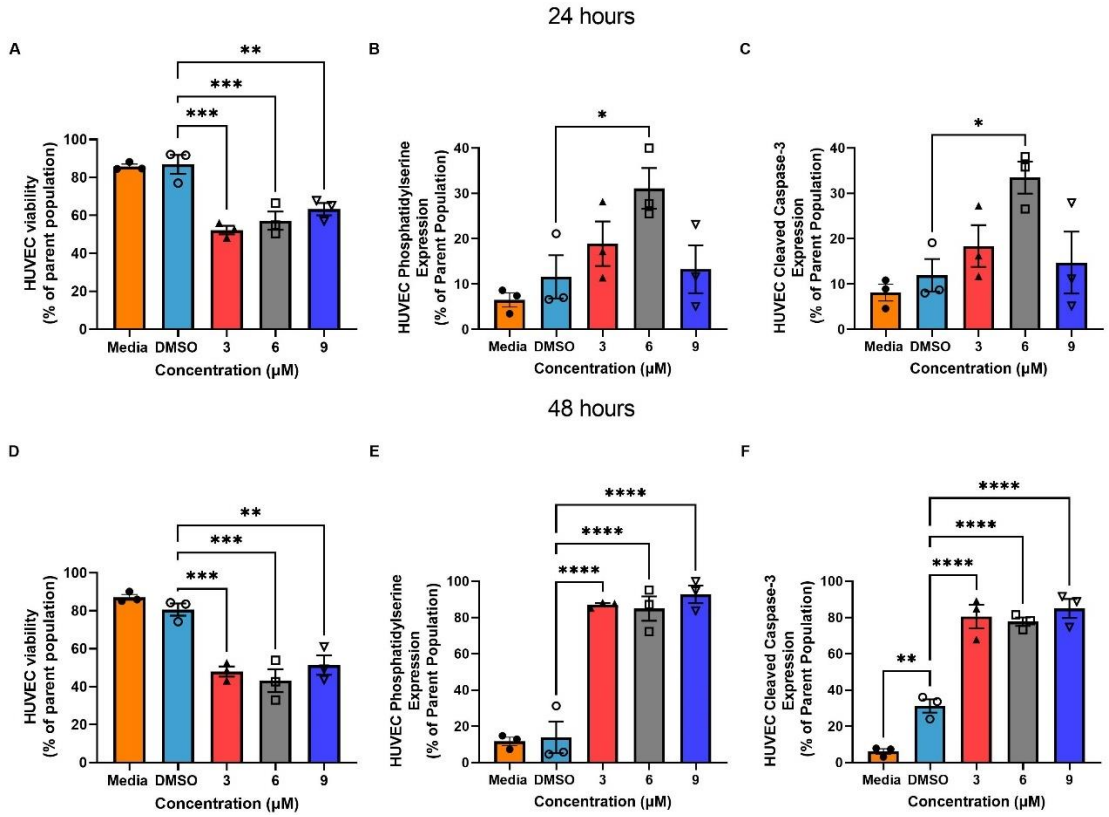

Figure 4.8. Dose-response effects of docetaxel on HUVEC cell viability (A, D), phosphatidylserine (B, E) and cleaved caspase-3 (C, F) expression, across a range of physiological concentrations at 24- (A, B, C) and 48-hours (D, E, F), as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001.*

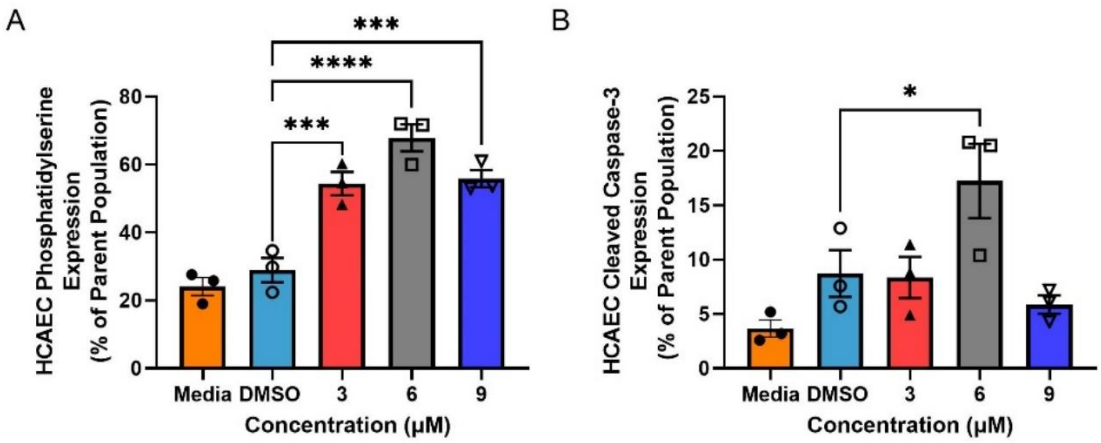

Figure 4.9. Dose-response effects of docetaxel on HCAEC phosphatidylserine (A) and cleaved caspase-3 (B) expression across a range of physiological concentrations at 48-hours post-exposure, as determined by ANOVA. *Data shown are mean ± SEM (n=3). ** p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.3.3 Dose Response Effects of FEC-T Drugs on Endothelial eNOS and Phospho-eNOS Expression

FEC exposure had significant effects on eNOS expression at 4- and 12-hours (**Figure 4.10A and C**). FEC exposure had a less definitive effect on phospho-eNOS, with no significant difference between-groups for phopsho-eNOS expression after exposure to physiological FEC concentrations (**Figure 4.10B and D**). Full results are displayed in **Appendix 9**. For HCAECs eNOS expression, FEC exposure had no significant effect across the range of FEC dosages (**Figure 4.11A**). This is in contrast to docetaxel exposure which showed a dose-dependent effect of docetaxel, reducing eNOS expression by 32% (**Figure 4.11B**). Full results are displayed in **Appendix 9.**

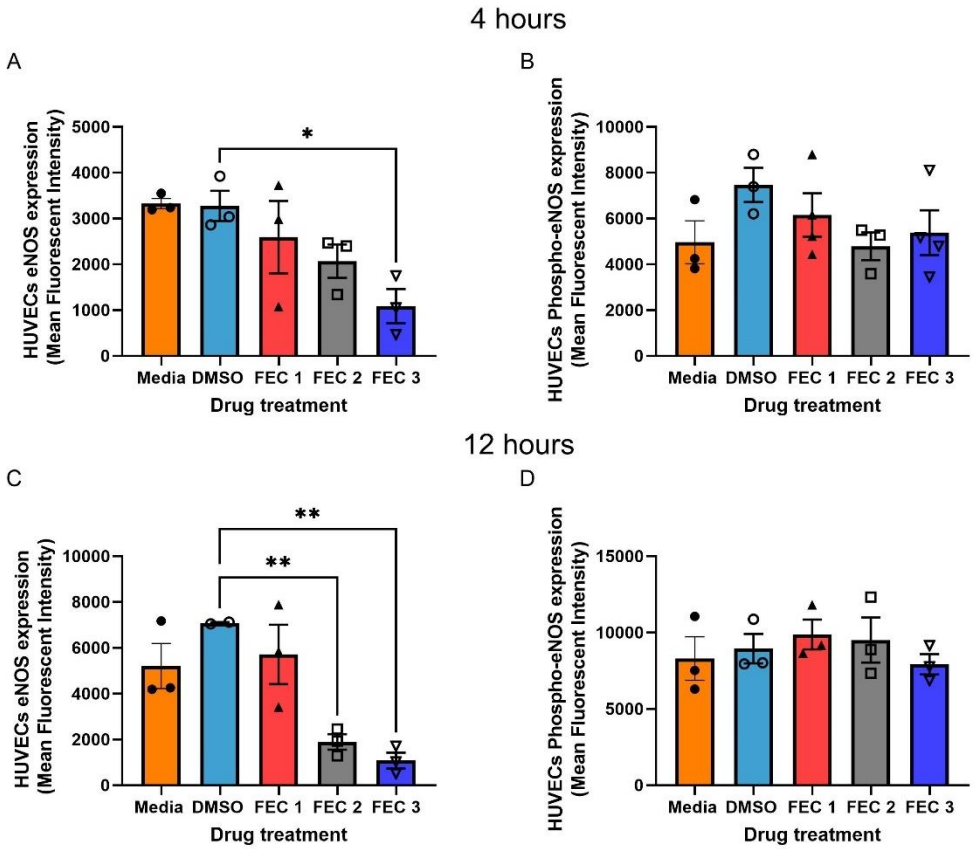

Figure 4.10. Dose-response effects of combined 5-fluorourcil, epirubicin and cyclophosphamide (FEC) on HUVEC eNOS expression (A, C) and eNOS activation state (B, D) at 4- (A, B) and 12 hours (C, D) across a range of physiological concentrations (FEC 1, FEC 2, and FEC 3), as determined by ANOVA. *Data shown are mean ± SEM (n=3). ** p < 0.01*

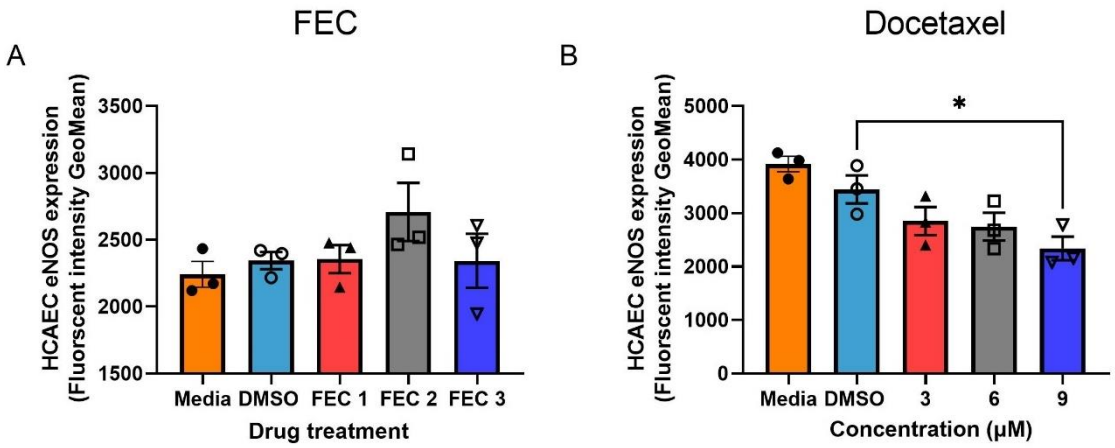

Figure 4.11. Dose-response effects of combined 5-fluorourcil, epirubicin and cyclophosphamide (FEC: A) and docetaxel (B) on HCAEC eNOS expression across a range of physiological concentrations (FEC 1, FEC 2, and FEC 3) at 4- and 48-hours, respectively, as determined by ANOVA. *Data shown are mean ± SEM (n=3). * p < 0.05 ** p < 0.01.*

4.3.4 Dose Response Effects of FEC-T Drugs on Endothelial Adhesion Molecule Expression

Interestingly, during preparation for analysis by flow cytometry, endothelial cells exposed to chemotherapy were increasingly difficult to detach from the surface of the well plate. It can be speculated that this is due to an increase in cell adhesion marker expression, causing an increase in strength of cellular adherence, as many pathological stimuli trigger activation of endothelial cells to express adhesion markers (Wu, Liu, & Zhou, 2017). Therefore, expression of adhesion molecule ICAM-1 was assessed in HCAECs. ICAM-1 was significantly reduced by FEC exposure, in a dose-dependent manner from 2025 ± 274 MFI to 431 ± 80 MFI (p = 0.0002) (**Figure 4.12A**). For HCAEC docetaxel exposure, there was also a dose-dependent decrease in ICAM-1 expression from 1849 ± 107 MFI to 877 ± 65 MFI (p = 0.0006) (**Figure 4.12B**).

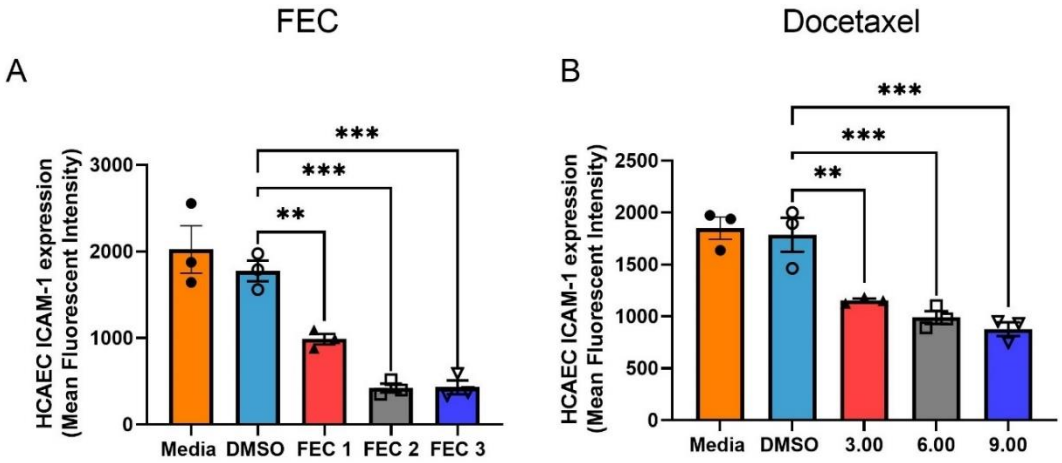

Figure 4.12. Dose-response effects of combined 5-fluorourcil, epirubicin and cyclophosphamide (FEC) (A) and docetaxel (B) on HCAEC ICAM-1 expression at 4- and 48-hours, respectively, hours across a range of physiological concentrations (FEC 1, FEC 2, and FEC 3), as determined by ANOVA. *Data shown are mean ± SEM (n=3). ** p < 0.01, *** p <0.001.*

4.3.5 Effects of FEC-T drugs on Endothelial Wound Healing

To investigate the impact of chemotherapy drugs on endothelial repair, a wound healing assay was performed. Representative images of the wound healing assays are displayed in **Figure 4.13**. Note that proliferation was not inhibited in this assay and therefore, wound closure is likely a function of cellular migration-proliferation, and not migration only as often reported in the literature (Martinotti, Calabrese, & Ranzato, 2017). Hence, the rate of wound closure is defined as the average velocity at which the cells collectively move into the gap. 5-FU (p = 0.0007), epirubicin (p = 0.015), docetaxel (p = 0.00002), and FEC combined (p = 0.0008) chemotherapy drugs significantly reduced the rate of gap closure in the scratch assay model of wound healing. There was no significant effect of cyclophosphamide on the rate of gap closure (p = 0.072). However, further analysis using $t_{1/2gap}$ revealed a significantly longer time for the wound to reach half of its initial size when compared to the no drug control (20.25 ± 3.38 hours vs. 10.44 ± 0.56 hours, p= 0.021). For HCAECs, wound healing the rate of gap closure was significantly reduced with all FEC-T drugs individually (5-FU, p = 0.0006; epirubicin, p = 0.024; cyclophosphamide, p = 0.006; docetaxel, p = 0.0007) and with combined FEC exposure (p = 0.0001).

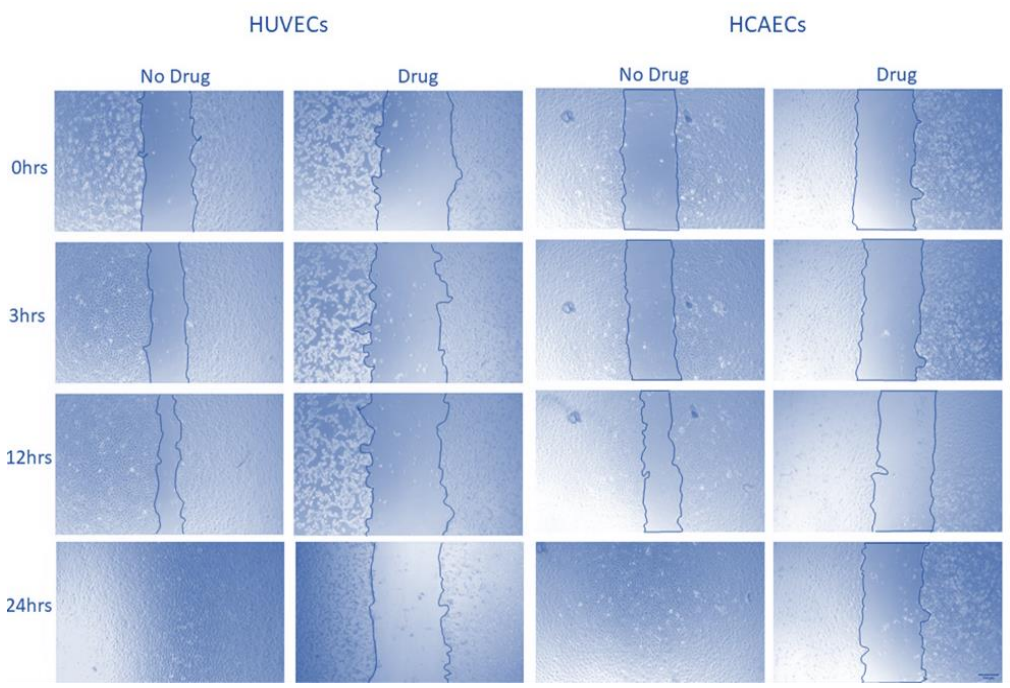

Figure 4.13. Representative image of HUVEC and HCAEC gap closure over time with and without drug exposure. *Images are x 4 magnification. Scale bar is 250µm.*

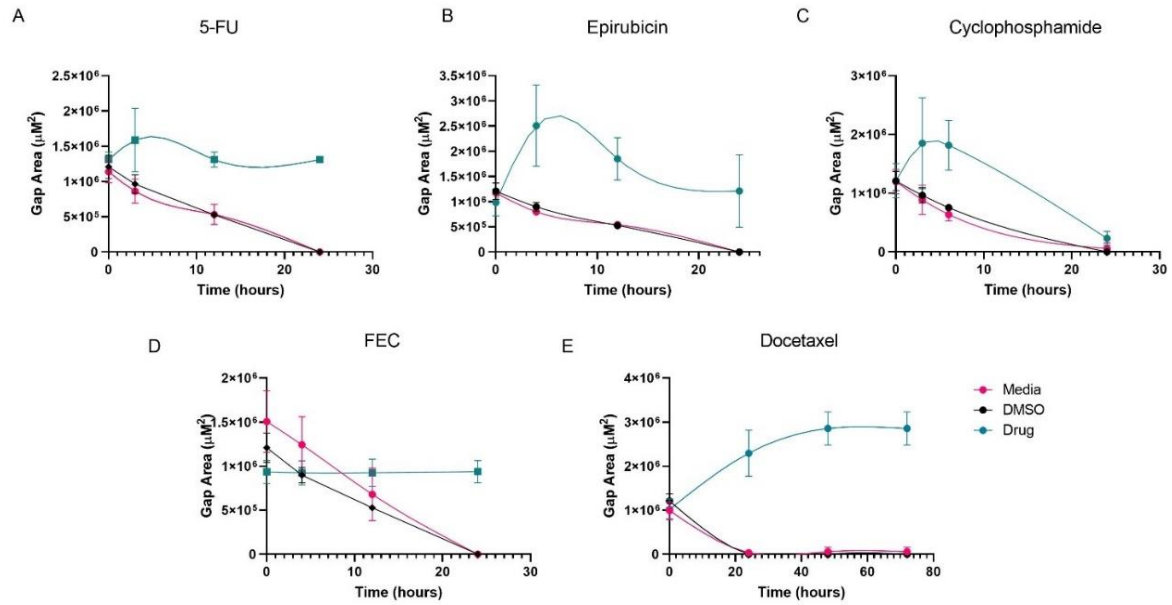

Figure 4.14. HUVEC wound closure (Gap area (μM^2)) over time (hours) with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), FEC combined (D), and docetaxel (E). *Data are presented as Mean ± SEM (n=3).*

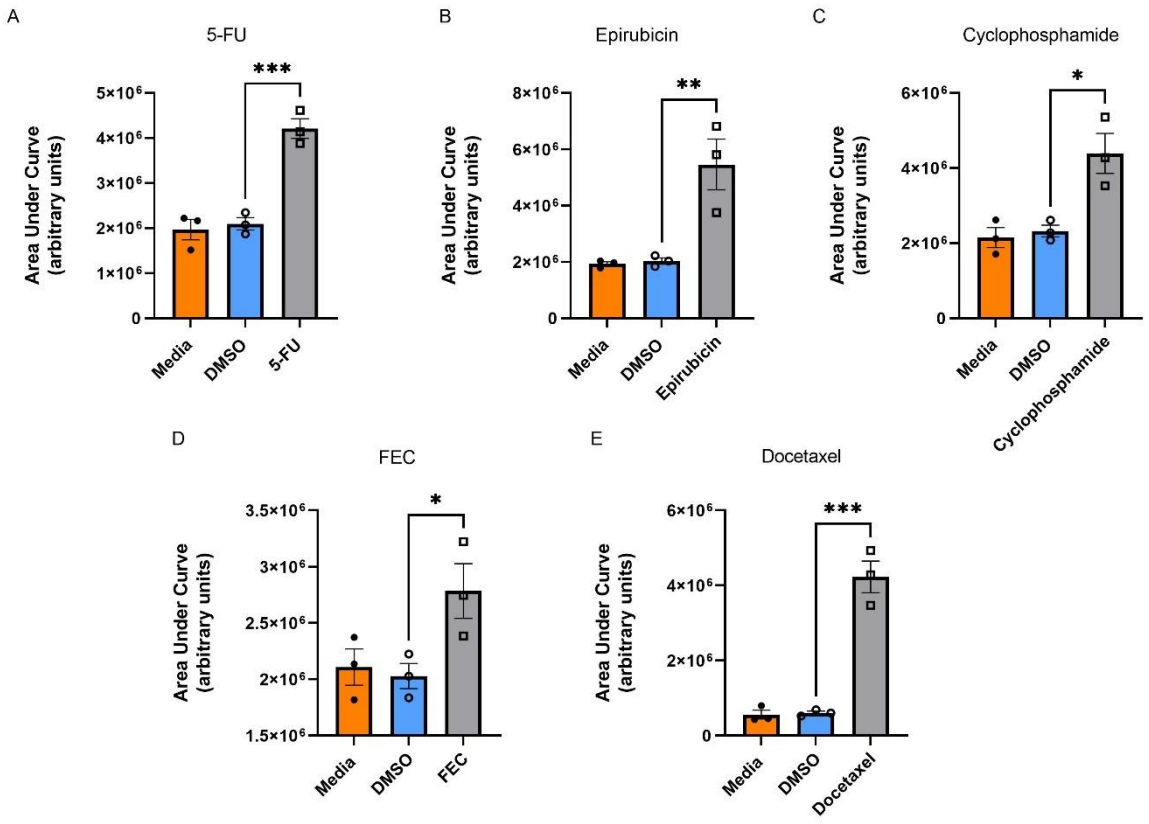

Figure 4.15. Area under the curve of HUVEC wound closure (Gap area (μM^2)) over time (hours) with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), FEC combined (D), and docetaxel (E), as determined by ANOVA. *Data shown are mean ± SEM (n=3). * p < 0.05, ** p < 0.01, ***p < 0.001.*

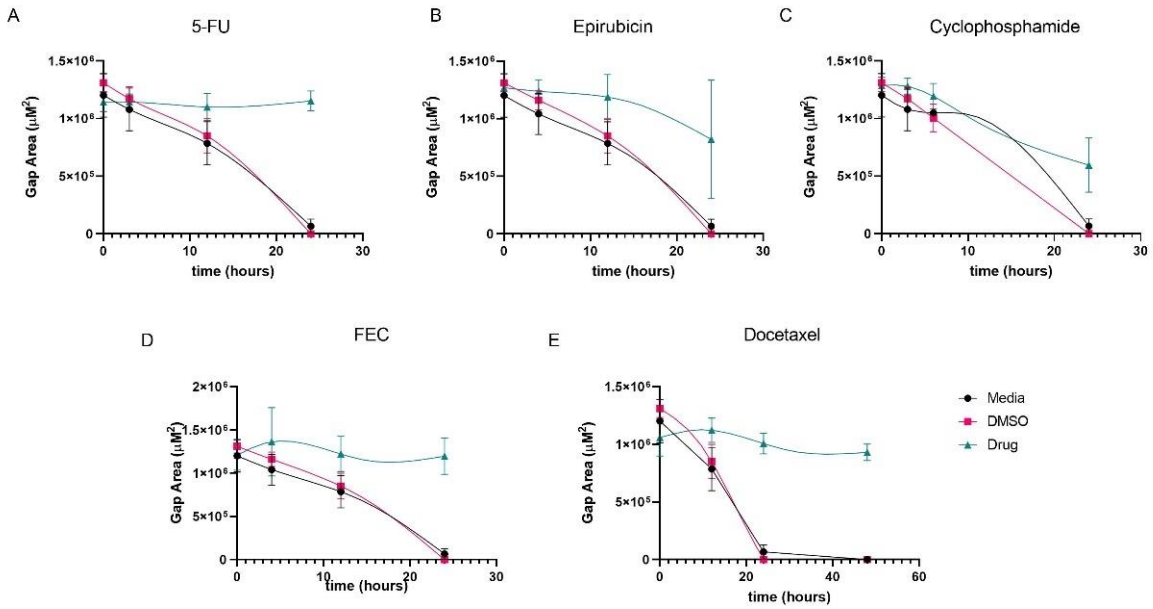

Figure 4.16. HCAEC wound closure (Gap area (μM^2)) over time (hours) with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), FEC combined (D), and docetaxel (E), as determined by ANOVA. *Data shown are mean ± SEM (n=3).*

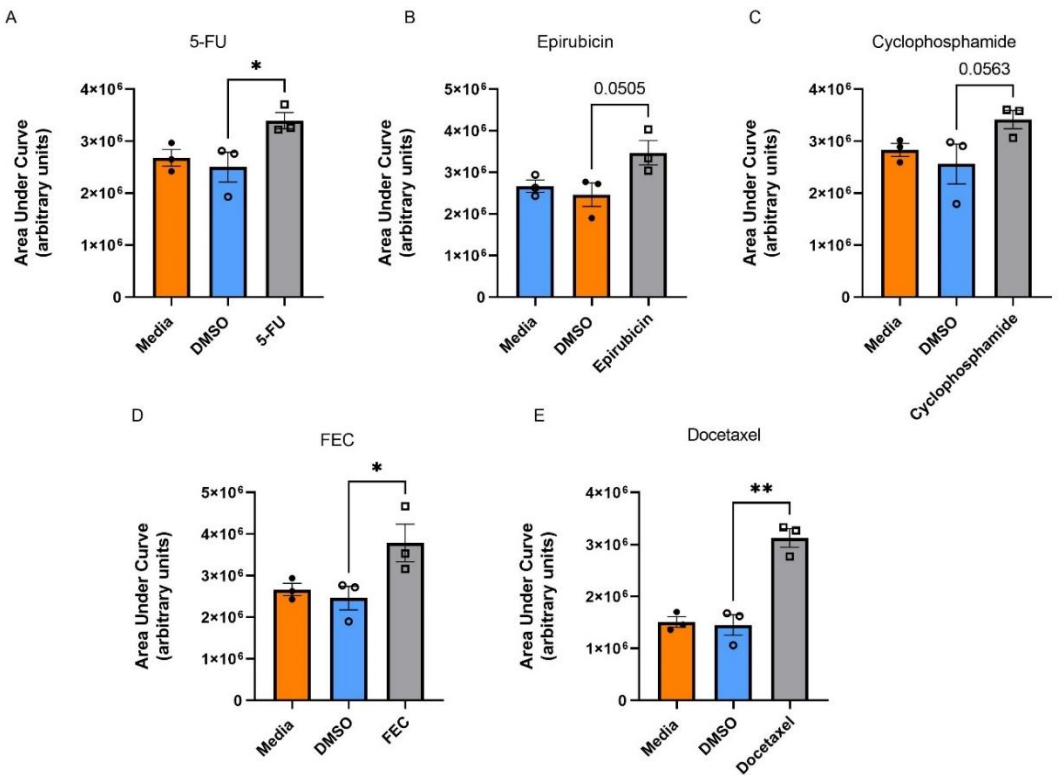

Figure 4.17. Area under the curve of HCAEC wound closure (Gap area (μM^2)) over time (hours) with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), FEC combined (D), and docetaxel (E), as determined by ANOVA. *Data shown are mean ± SEM (n=3). * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.*

4.4 Discussion

The aim of the current investigation was to determine the effects of FEC-T chemotherapy treatment for BC on the viability and function of endothelial cells using HUVECs and HCAECs as *in vitro* models. The main finding is that there is a strong dose-response effect of FEC-T drugs both individually and combined (FEC) on endothelial cell viability, with dose-dependent increases in cleaved caspase-3 and Annexin V binding to phosphatidylserine. With regards to endothelial cell function, overall effects of chemotherapy on eNOS were a reduction in content with no real effect on levels of phosphorylation. Furthermore, FEC-T drugs appear to affect the usual expression of endothelial cell adhesion molecules, with downregulation of ICAM-1. The current findings on endothelial cell viability and function have valuable physiological significance which can be added to the literature.

4.4.1 Effects of FEC-T drugs on Endothelial Cell Viability

The suspected detrimental effects of BC chemotherapy treatment on the endothelium can be visualised by the development of shrunken and detached cells (**Figure 4.2**), indicating an induction of cell death with FEC-T treatment. To confirm this, chemotherapy effects on endothelial cell health were assessed using biomarkers of cell death and apoptosis which were quantified using flow cytometry. For each FEC-T drug, there was a dose-dependent but not a time-dependent reduction in HUVEC cell viability (with exception to docetaxel which showed similarly detrimental effects on viability across dosages), which occurred alongside an increase in Annexin-V binding to phosphatidylserine (suggestive of early cell-apoptosis) and cleaved caspase-3 expression (late apoptotic marker) (**Figures 4.3-6, 8**). These results are paralleled in HCAECs with exposure to FEC (**Figures 4.7**) and docetaxel (**Figures 4.9**). This suggests that the reduced cell viability occurs alongside caspase-3 mediated apoptosis. This is the case for all the individual FECT drugs commonly used for BC treatment, suggesting each individual drug and FEC combined treatment is toxic to the vasculature. Each FECT drug has its own individual mechanism of action, including damaging DNA (Chighizola et al., 2011; Longley et al., 2003) and RNA (Longley et al., 2003); inhibiting cellular enzymes (Stěrba et al., 2013) and tubulin (Gligorov & Lotz, 2004) to prevent DNA synthesis; and increasing oxidative stress (Stěrba et al., 2013) to create a non-functional cellular environment. Regardless of the mediating mechanism, each drug ultimately leads to activation of a caspase cascade which

induces cellular apoptosis (Antonella De Angelis et al., 2017; Chighizola, Ong, & Denton, 2011; Gligorov & Lotz, 2004; Stěrba et al., 2013).

Other *in vitro* studies of the effects of chemotherapy exposure on endothelial cells have had similar findings to the current study. Exposure to 3 or 5 $\mu g \cdot ml^{-1}$ epirubicin for 30 minutes caused reduced HUVEC cell viability (Eakin et al., 2020). Porcine aorta endothelial cells exposed to 3-30μM epirubicin decreased cell viability and increased caspase-3 and 7 activity in a dose- and time-dependent manner (Yamada et al., 2012). Similarly, an EA.hy926 immortalised human endothelial vein cell line exposed to $100\mu g \cdot ml^{-1}$ 5-FU for 4 hours significantly increased endothelial cell death (Altieri et al., 2017). Likewise, prolonged exposure (144 hours) to low-dose chemotherapeutic drugs paclitaxel (taxane chemotherapy drug) and 4-hydroperoxycyclophosphamide both increased HUVEC cell apoptosis by >3-fold compared to no drug control (Bocci, Nicolaou, & Kerbel, 2002); and HUVEC exposure to doxorubicin (anthracycline drug) results in significant apoptosis (Wu et al., 2002). Doxorubicin reportedly associated with endothelial cell loss in an *ex vivo* study of rat aorta (Bosman et al., 2021). The current study adds to the literature that this is also the case for endothelial cells exposed to physiologically relevant concentrations of FEC-T drugs for BC.

These *in vitro* findings correspond to *in vivo* work which shows that chemotherapy increased CECs by 3-fold in BC patients after just a single dose, indicating acute endothelial injury (Beerepoot et al., 2004). This reduction in endothelial cell viability has implications for vascular health, with endothelial insult without efficient repair being one of the mediating mechanisms behind the initiation of vascular disease and CVD mortality (Werner et al., 2008). Chemotherapy-induced endothelial injury initiates neutrophil and monocyte adhesion to damaged endothelium (Cameron, Touyz, & Lang, 2016) and likely increases the uptake of low-density lipoprotein and recruitment of white-blood cells into the tunica intima, leading to development of atherosclerosis (Sullivan, Sarembock, & Linden, 2000), subsequently leading to hypertension, MI and stroke (Insull, 2009). This endothlial cell damage and cell death therefore, may explain the increase in venuous threomboembolic events with chemotherapy treatment (Petrelli, Borgonovo, Cabiddu, Lonati, & Barni, 2012). Furthermore, damage to the endothelium in the coronary vasculature results in leakage of toxic metabolites which directly damage cardiomyocytes (Pai & Nahata, 2000). This may create an access route for other chemotherapy drugs into the heart, contributing to

cardiotoxicity. Therefore, without efficient repair, endothelial cell death may contribute to chemotherapy-induced CV toxicity which, depending upon treatment type and dosage, has an incidence of up to 9% in BC patients (Yeh, Courtney, & Bickford, 2009), leading to a CV mortality rate of ~16% (Patnaik et al., 2011). Our findings that chemotherapy exposure significantly reduces endothelial cell viability (**Figure 4.3-7**) strengthens the evidence that cancer treatment toxicities occur in the vasculature and that this is a cause for concern for the field of oncology medicine.

4.4.2 Effects of FEC-T Drugs on eNOS/Phospho-eNOS

Endothelial function was also investigated in the current study by measuring eNOS expression and phosphorylation. For HUVECs there is a dose- and time-dependent effect of FEC combined drug exposure on eNOS expression (**Figure 4.10A and C**). However, in HCAECs, there is no significant effect of FEC on eNOS expression (**Figure 4.11A**), but docetaxel exposure caused endothelial dysfunction as demonstrated by significantly reduced eNOS expression in a dose-dependent manner (**Figure 4.11B**). It is perhaps important to note that at both 4 and 12-hours there is non-significant reduction in phosphorylation of eNOS in HUVECs (**Figure 4.10B and D**). Another *in vitro* study – investigating 5-FU and epirubicin – found that eNOS and phospho-eNOS both reduced with chemotherapy exposure (Gajalakshmi et al., 2013). Similarly, reduction in eNOS bioavailability and activation occurs alongside cell damage with 5-FU and epirubicin exposure (Gajalakshmi et al., 2013). Cisplatin (an alkylating agent similar to cyclophosphamide) has also been found to reduce eNOS content in HUVECs (Sekijima et al., 2011).

Reduction in eNOS bioavailability and activation has major implications for vascular function and consequently increases the risk of CVD (Benjamin et al., 2004). This key enzyme is required for vasodilation and preventing atherosclerosis development via inhibition of endothelial-leukocyte binding (Cooke & Tsao, 2011). Looking to relevant *in vivo* studies, it has been found that the expected reduction in vascular function with chemotherapy treatment, is in fact the case. Duquaine et al. (2003) found that a single dose of anthracycline chemotherapy reduced FMD in BC patients from 6.5%, typically seen in healthy populations (Ghiadoni et al., 2012), to 2.5%, typically seen in CVD populations

(Benjamin et al., 2004). Similarly, FMD reduced in 27 women with BC treated with taxanes (paclitaxel: 6.2% to 2.2%; paclitaxel plus anthracycline: 5.6% to 0.3%) (Vassilakopoulou et al., 2010). The current findings support these findings by providing insight that FMD may be reduced due to reduction in endothelial cell eNOS expression with chemotherapy exposure. Therefore, the difference in endothelial health between cancer and non-cancer patients is not simply the cancer itself but the treatment process, and hence, endothelial cells do not need to be derived from cancer patients to be relevant to the question of chemotherapy exposure effects. The implications of a chemotherapy-induced reduction in eNOS activity and subsequently impaired ability for vasodilation is of significant clinical importance. The level of FMD found in BC patients after chemotherapy treatment is equivalent to that typically seen in CVD patients (Benjamin et al., 2004) and is likely a driver of CV toxicity. FMD is predictive of chemotherapy cardiotoxicity, with every 2.7% increase in FMD associating with a 37% less likelihood for LVEF reduction at 3 months post-chemotherapy treatment in BC patients (Anastasiou et al., 2017). Interestingly, Chong et al. (2004) found an inverse correlation between CECs and FMD (r = -0.423; p = 0.002), suggesting that vascular endothelial injury is perhaps also responsible for a reduction in vascular function. Doxorubicin-induced endothelial cell death associated with reduced eNOS expression in an *ex vivo* study of rat aorta, relating to arterial stiffness (Bosman et al., 2021). This could also be the reason for the reduction in eNOS bioavailability as with more dead cells, there would be less eNOS production. Furthermore, as endothelial cells undergo apoptosis, eNOS expression was also reduced. This is in agreement with the literature that alongside a 3-fold increase in apoptosis of human aortic endothelial cells, eNOS and phospho-eNOS expression both decreased by ~50% (Rippe et al., 2012). Interestingly, eNOS also prevents cellular apoptosis (Ho et al., 2006), therefore, both endothelial damage and vascular dysfunction go hand-in-hand. A reduction in vasodilatory capacity through reduced eNOS production and activation results in systemic hypertension, increased risk of thrombosis (Antonella De Angelis et al., 2017), and is likely a mechanism contributing to the 5% incidence of HF associated with chemotherapy (Swain, Whaley, & Ewer, 2003b). It is likely that chemotherapy-induced vascular dysfunction in BC patients is due to a combination of reduction in NO production and cell death, based on current findings that FEC reduces eNOS content in HUVECs by up to 90% (**Figure 10C**) and docetaxel reduces eNOS content of

HCAECs by up to 40% (**Figure 4.11B**), occurring alongside ~30% reductions in viability (**Figure 4.6A**).

4.4.3 Effects of FEC-T Drugs on Endothelial Cell Adhesion Molecule Expression

As well as eNOS expression and phosphorylation, analysis of adhesion molecule expression has also revealed some interesting novel findings, with both FEC and docetaxel treatments significantly reducing ICAM-1 expression by 79% and 53%, respectively (**Figure 4.12**). Regulation of adhesion molecule expression is an important function of endothelial cells. ICAM-1 is important in adhesion of leukocytes to the activated endothelium, consequently leading to build up of macrophages within the intima (Wu, Liu, & Zhou, 2017b). These are crucial steps in the process of atherosclerosis development and hence, adhesion molecule regulation is essential for endothelial cell control over plaque risk (Wu et al., 2017b).

The downregulation of ICAM-1 in this study was unexpected and contrasts the literature, with *in vitro* and *in vivo* animal studies having found that both alkylating agents and taxanes induce upregulation of ICAM-1 (Soultati et al., 2012a). Firstly, previous *in vitro* studies found that taxane exposure increases ICAM-1 expression and induces apoptosis (as measured by flow cytometry) (Meijer, 2009). The difference in our results are yet to be fully explained but this may be due to the different incubation times. Meijer *et al*. (2009) anlaysed endothelial cells at 24-, 48- and 72-hour time points. The latest time-point in the current studies was 12-hours and hence, this may be an effect which occurs onwards of the 12 hour time-point utilsed in the current anaysis. Furthermore, *in vivo* studies have found that chemotherapy drugs increase circulating levels of ICAM-1 in cancer patients (Baar et al., 2009). Again, an increase in inflammation with chemotherapy is a probable explanation for increased ICAM-1 expression as molecules such as TNF-α stimulate expression of adhesion molecules (Wu et al., 2017b). This increases the risk of atherosclerosis (Silvestro, Brevetti, Shiano, Scopacasa, & Chiariello, 2005) and may play a role in the increased CVD incidence associated with breast cancer treatment (Patnaik, Byers, DiGuiseppi, Dabelea, & Denberg, 2011). However, the current findings did not confirm this *in vitro* effect. It is important to consider that results from *in vitro* experiments are not directly comparable with *in vivo* measurements of soluble adhesion molecules as these molecules may be derived from sources other than endothelial cells (Mills et al., 2004). That said, there is now evidence that the initial effect of

chemotherapy is to reduce soluble ICAM-1 levels in cancer patients. Compared with pre-treatment, sICAM-1 levels were decreased at week 2 of chemotherapy cycles (Mills et al., 2004). However, at the start of cycle 4, sICAM-1 levels were elevated (Mills et al., 2004). It may in fact be the case that the more immediate effect of chemotherapy is to reduce sICAM-1, with continued treatment leading to the elevations reported in other chemotherapy studies (Baar et al., 2009). This would explain our *in vitro* findings as there was only one chemotherapy exposure. Hence, assays that are able to include repeated endothelial cell exposures to chemotherapy are required to confirm this phenomenon *in vitro*. Additionally, in a study of photodynamic therapy, endothelial ICAM-1 expression is upregulated at a transcriptional level but is downregulated at a protein level due to degradation by lysosomal proteases (Volanti et al., 2004). This may well be occurring in the current study as intracellular ICAM-1 expression may be upregulated by chemotherapy stimuli, but this does not reach endothelial cell surface expression as the adhesion molecules are lost before they can be expressed. This is plausible as there is increased cell death with chemotherapy exposure which may lead to apoptotic blebbing (Navratil, Watkins, Wisnieski, & Ahearn, 2001) or lysosomal proteases degradation (Volanti et al., 2004) leading to loss of extracellular adhesion molecule expression. Therefore, this study should be repeated using intracellular measurements of ICAM-1 as well as cell surface expression.

4.4.4 Effects of FEC-T Drugs on Endothelial Repair

Our wound healing assay results show that the ability of HUVECs and HCAECs to migrate and proliferate across a mechanically produced gap is significantly impaired with exposure to FEC-T drugs. Usually, upon damage, endothelial cells near the gap edge sense the insult/wound and, with stimulation from growth factors, initiate directed migration to fill the wound area (Michaelis, 2014). This process allows maintenance of endothelial integrity and reduces atherosclerotic risk, with endothelial injury being the initiating step in vascular disease (Cooke & Tsao, 2011; Insull, 2009). This process is neatly followed in the control cells which were not exposed to any drugs (**Figure 4.13-17**). However, is inhibited with exposure to all FEC-T drugs (**Figure 4.13-17**). This is in agreement with the literature whereby a meta-analysis found that chemotherapy drugs inhibit endothelial cell migration and proliferation *in vitro* (Soultati et al., 2012a). Cell death was substantial in some of the assays, so much so

that the wound did not heal by the end of the 24-48-hour timeframe, and in fact, increased in size instead. This effect is determined by the observation of a significant increase in floating/dead cells which occurred alongside the increases in gap size. It can be assumed that the increase in gap size is therefore due to dead cells breaking off from around the injury site. Therefore, not only was migration impaired, but the injury was worsened due to chemotherapy-induced cell death increasing the area size. As this was a measure of wound healing and not of cell death, this was corrected in the analysis of rate of gap closure by giving a value of 0.00μm·hr^{-1} to these samples. It is probable that this is linked to the increasing cell death that is observed in the flow cytometry assays as induction of apoptosis occurs alongside a reduction in cell migration (Yao et al., 2015), likely occurring via the p38/MAPK signalling pathway (Martínez-Limón, Joaquin, Caballero, Posas, & de Nadal, 2020; Yao et al., 2015; Yu et al., 2004). Furthermore, chemotherapy-induced impairment of endothelial migration, as assessed by wound healing assay, has been shown previously to be related to reduced NO production (Gajalakshmi et al., 2013). Regardless of the mechanism, we have demonstrated that the usual endothelial repair process which protects against atherosclerotic development is lost with chemotherapy exposure, likely contributing to the atherosclerotic risk observed in those treated with chemotherapy for breast cancer (Cameron, Touyz, & Lang, 2016b).

4.5 Conclusions and Implications

FECT-induced endothelial damage and dysfunction occurs at a cellular level, with an increase in cellular apoptosis, reduced eNOS expression and function, altered adhesion molecule expression, and an inhibition of wound repair. The importance of the role of the endothelium in CV toxicity is clear, and there is evidence for all FEC-T drug treatments causing detriments to the vascular endothelium. Therefore, precautions to protect the vasculature and/or detect, manage, and repair the damage should be taken when undergoing any chemotherapy regime involving FECT drugs to reduce the implications of vascular toxicity in BC patients.

Chapter 5

The protective effects of habitual physical activity status on vascular endothelial cell toxicity of FEC-T chemotherapy treatment

5.1 Introduction

As shown in *Chapter 4*, FEC-T chemotherapeutics induce several toxic effects on vascular endothelial cell, including (1) increased apoptosis, (2) reduced eNOS and (3) ICAM-1 expression, and (4) an impaired ability to heal wounds. These toxicities may contribute to the cardiovascular detriments that chemotherapy treatment causes in BC patients (Patnaik et al., 2011a). Strategies to alleviate this toxicity are required for disease-free survival. Interestingly, there is evidence for physical activity/exercise protection against cardiovascular toxicity in BC survivors, with those who are physically active having a reduced risk of developing cardiovascular toxicity with chemotherapy (Lee W Jones et al., 2016; Nagy et al., 2017b). The mechanism behind this effect potentially involves direct effects on the endothelium. Physical activity/exercise has been demonstrated to improve vascular endothelial health and function in clinical populations, including CAD patients. These improvements include enhanced vascular endothelial function as a result of greater eNOS protein expression in endothelium (Hambrecht et al., 2003). The higher eNOS content and activation, together with enhanced vascular relaxation, indicates strong protective effect of exercise in at-risk populations. These adaptations are highly linked to shear stress (the effect of blood flow across the endothelial cell membranes) (Tinken et al., 2010), however, there may be systemic adaptations which may be favourable for endothelial health, such as altered lipid profiles (Beazer et al., 2020; Durham, Chathely, Mak, et al., 2018; Kodama et al., 2007; Mineo, Deguchi, Griffin, & Shaul, 2006), and reduced fasting glucose (Devan et al., 2013; Gillen et al., 2012). Due to the impact regular exercise has on endothelial health, it may be a protective strategy which could be used to alleviate or attenuate chemotherapy-associated vasculotoxicity.

Therefore, the potential for exercise-induced protection of the vascular endothelium should be investigated. The aim of this study is to investigate whether physical activity status can attenuate any deleterious effects of chemotherapy on endothelial cells. To achieve this aim, an *in vitro* model of serological preconditioning was also developed, whereby serum from physically active and inactive individuals were used in cell culture model prior to exposure to chemotherapy. Endothelial health was then assessed using the same outcome measures as in *Chapter 4* (cell viability, apoptosis, caspase-3 activation, eNOS content and adhesion

molecule expression). It is hypothesised that there will be a protective effect of preconditioning the cells with serum from habitually active individuals compared with inactive individuals.

5.2 Methodology

5.2.1 Participants

Participants were recruited based on the following inclusion criteria: healthy females, Caucasian, 18-35 years, BMI 18-24.9kg·m^2. Participants were excluded for presence of diabetes, neurological conditions, hypertension, or CVD (cardiomyopathy, angina, coronary artery disease, arrhythmias). Participants were classed as "physically active" or "inactive" based on results from self-reported physical activity, measured by The International Physical Activity Questionnaire (>150 minutes/week moderate-vigorous physical activity (MVPA) = "active", <120 minutes/week MVPA = "inactive").

Ethical approval was granted from Edinburgh Napier University Research Integrity Committee and all participants provided written informed signed consent before participation in this study.

5.2.2 Serum Collection

On the day of testing, participants were fasted, having participated in no exercise and no consumption of caffeine, drugs, or alcohol within the last 24-hours. Venous blood (14ml) was collected from the antecubital vein in serum tubes (BD Bioscience, USA, catalogue number: 367812). Samples were allowed to clot at room temperature (~30 minutes), centrifuged at 900 x *g* for 10 minutes, and the serum carefully removed from top layer using a pistette. Samples were stored at -20°C until use in the *in vitro* model. Once all serum was collected, samples were pooled based on physical activity status, creating one physically active and one inactive sample. This is to limit inter-individual variability.

5.2.3 Fitness Test

Directly after blood draws, a graded incremental exercise test to exhaustion was carried on a treadmill (Woodway, ERGO ELG-55, Germany) to quantify peak oxygen consumption ($\dot{V}O_{2peak}$). Breath-by-breath analysis was conducted using a Cortex Metalyzer 3B (Cortex

Biophysyik GmbH, Germany) and analysed using MetaSoft Studio version 5.8.6 SR1 (Cortex Biophysyik GmbH 2016, Germany). $\dot{V}O_{2peak}$ was determined as the average $\dot{V}O_2$ output of the last 30 seconds of the test or as the maximal plateau whereby the $\dot{V}O_2$ output was consistent for 4 consecutive breaths (Barker et al., 2011; Rossiter et al., 2006).

5.2.4 *In Vitro* Methodology

5.2.4.1 Endothelial Cell Serum Preconditioning

Unpooled human umbilical vascular endothelial cells (HUVECs) (product code: C0035C, Thermofisher Scientific, USA) and human coronary artery endothelial cells (HCAECs) (product code: 300-05A, Sigma-Aldrich, Gillingham, UK) were cultured in endothelial cell growth medium (ECGM; product code: 211-500, Sigma-Aldrich, Gillingham, UK) and Meso-endothelial cell growth medium (MEGM; product code: 212-500, Sigma-Aldrich, Gillingham, UK), respectively, supplemented with 5% pooled active or inactive serum (Zitta et al., 2012), and incubated at 37°C in 5% CO_2 for 24-hours.

5.2.4.2 Endothelial Cell Chemotherapy Exposure

Appropriate concentrations of 5-fluorouracil (product code: ab142387; Abcam, UK), epirubicin hydrochloride (product code: ab142100; Abcam, UK), cyclophosphamide (product code: ab141240, Abcam, UK) and docetaxel (product code: ab141248; Abcam, UK) were added to the appropriate corresponding wells, individually and as combined FEC, as in *Chapter 4*. Drug concentrations were based on the serum concentration found in patients after exposure to each of these drugs (5-FU: 1.5µM (Reigner et al., 2003); epirubicin: 0.006µM (Danesi et al., 2002); cyclophosphamide: 38µM (Adams et al., 2014); and docetaxel: 6µM (Hurria et al., 2006)). Drugs were washed off at time-points corresponding to the literature findings when serum levels of each drug were diminished (Reigner et al., 2003);(Danesi et al., 2002); (Adams et al., 2014); (Hurria et al., 2006), and endothelial cells re-incubated at 37°C in 5% CO_2 until the appropriate analysis time-points, as in *Chapter 4*.

5.2.4.3 Sample Preparation for Analysis of Drug Effects

HCAECs were analysed at the most potent time-points: 5-FU: 3 and 12 hours; epirubicin: 4 and 12 hours; cyclophosphamide: 3 and 6 hours (Chow & Loo, 2003); and docetaxel: 24 and

48 hours (Morse et al., 2005); and for FEC: 4 and 12 hours. At the respective time-points, cells media was removed, and cells were washed with 200µl NaCl.

5.2.4.4 Flow Cytometry

Flow cytometry measured were performed, as detailed in *Chapter 4*, using the following mAbs: CD31-FITC (endothelial cell marker; product code: 303104; BioLegend, USA), Annexin V-PerCP-Cy5.5 (product code: 561431; BD Biosciences, USA), cleaved caspase-3-V450 (product code: 560627; BD Biosciences, USA) and eNOS-PE (product code: 130-106-787; Miltenyi Biotec, Bergisch Gladbach, Germany). A separate panel was used for analysis of ICAM-1 CD54-AlexaFluor 488 (product code: 322713, BioLegend, UK), as in *Chapter 4*. All samples were analysed using High Throughput System (HTS) on a 12-colour flow cytometer (FACS Celesta, BD Biosciences, USA).

5.2.4.5 Flow Cytometry Data Acquisition

Data were acquired using FACSDiva 6.0 Software (BD Bioscience, USA). Flow cytometer setup and calibration were performed using CS&T beads (BD Biosciences, USA). For sample acquisition, a minimum of 10,000 cell events were collected. Data were acquired using FACSDiva 6.0 Software (BD Bioscience, USA). Fluorescence compensation was performed as described in the Methodology and Materials chapter *(Chapter 3).*

5.2.4.6 Flow Cytometry Gating Process

Firstly, forward scatter and side scatter dot plots were created to determine the size and morphology of all events, allowing selection of the population of interest. A plot of forward scatter-area and -height was then created, gated on the population of interest. From this plot, singlets were selected. All subsequent plots were gated on singlets to remove any potential doublets from data analysis. FITC-CD31$^+$ expression confirmed endothelial cell identity. Once gated on CD31$^+$ cells, dot plots and histograms of annexin V-PerCP-Cy5.5, cleaved caspase-3-V450, and eNOS-PE could be visualised, and the data interpreted. ICAM-AlexaFluor 488 was analysed in the same way using a separate panel. Representative gating strategy is shown in **Figure 5.1**.

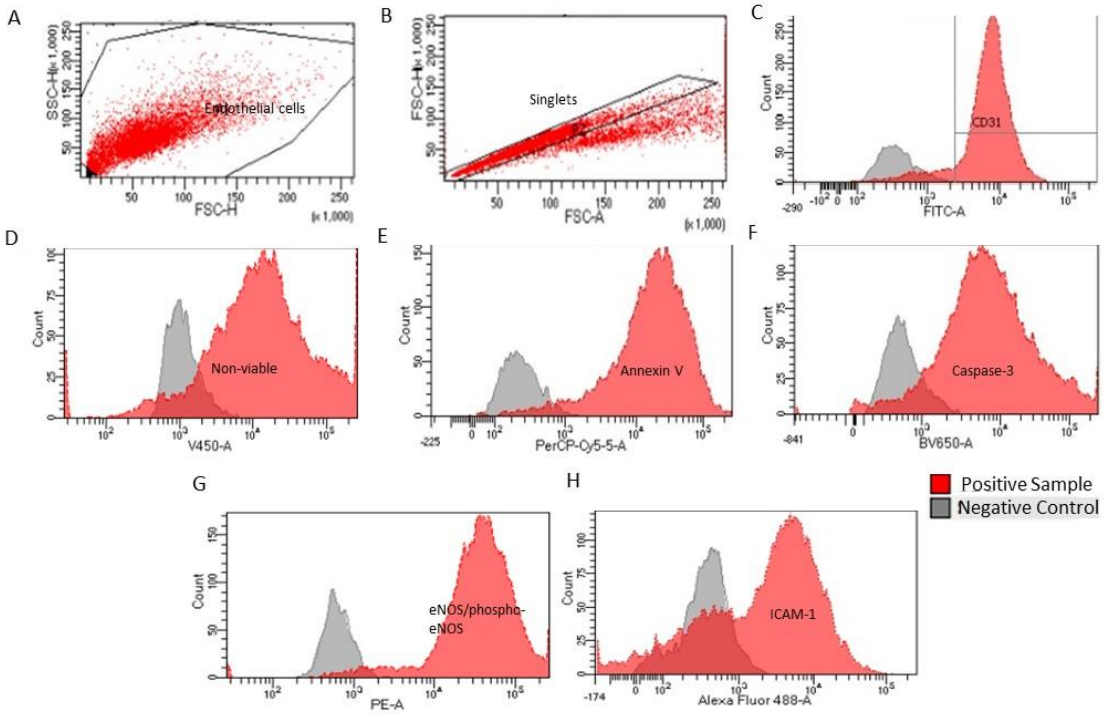

Figure 5.1. Representative gating strategy for HCAECs by morphology (A), singlets (B), and CD31 expression as displayed by FITC histogram (C). Histograms of Viability (D), Annexin V (E), cleaved caspase-3 (F), eNOS (G), and ICAM-1 (H) expression are displayed as a function of their respective lasers (PerCp-Cy.5, V450, PE, AlexaFluor488, respectively). *Histograms are displayed as GeoMean (Mean Fluorescent Intensity (MFI)).*

5.2.4.7 Wound Healing Assay

Endothelial cells were cultured in 24 well plates and treated with medium containing 5% habitually active or inactive serum and incubated at 37°C 5% CO_2. After 24-hours of serum preconditioning, the endothelial monolayer was inflicted with a wound and analysed, as described in *Chapter 4*. Rate of gap closure was analysed using image analysis software (Image J 1.x, Java, USA). After measuring the gap area for each image, gap area was plotted as a function of time to derive the rate of wound closure. For analyses where the rate of wound closure was not different between groups, the $t_{1/2gap}$ value (the point at which the gap is half the original area) was also calculated. Calculations for rate of wound closure and $t_{1/2gap}$ are as follows (Jonkman et al., 2014):

$$t1/2\text{gap} = \frac{\text{InitialGapArea}}{2 \times |\text{slope}|}$$

$$\text{Vwound closure} = \frac{|\text{slope}|}{2 \times \text{l}}$$

Area under the curve (AUC) was also calculated to create a visual representation of the change in the gap size over time.

5.2.5 Statistical Analysis

To assess between-group (active vs inactive) difference in participant characteristics, independent t-tests were performed using GraphPad Prism Version 9.0.1 (USA). All *in vitro* experiments were performed in biological triplicate and pooled, allowing statistical analysis to be performed. Shapiro-Wilks test of normality were performed. All data was determined to be normally distributed. One-way ANOVAs were performed using GraphPad Prism Version 9.0.1 (GraphPad Software Inc, USA) to determine differences between DMSO vehicle control, chemotherapy without serum conditioning (results taken from *Chapter 4*) and both serum conditions (active and inactive) on endothelial cell viability, apoptosis, eNOS, ICAM-1 expression and wound healing (AUC, $V_{migration}$, $T_{1/2\ gap}$) for each of the drug conditions (5-FU, epirubicin, cyclophosphamide and docetaxel, separately and FEC combination). Statistical significance was assumed if $p < 0.05$.

5.3 Results

5.3.1 Participant Characteristics

Six healthy females were recruited based on inclusion-exclusion criteria (active n=3, inactive n=3). Active and inactive were similar in age (habitually active: 25 ± 3 years vs 26 ± 4 years, p = 0.37). Body mass index (BMI) was also similar between groups (active: 23.97 ± 0.78kg·m^2 vs. inactive: 22.3 ± 1.84kg·m^2, p = 0.15). MVPA was significantly different between groups (active: 470 ± 61 minutes/week vs inactive: 98 ± 20 minutes/week; p = 0.0006). $\dot{V}O_{2peak}$ was significantly different between groups (active: 43.23 ± 3.56 ml/kg/min vs inactive: 35.36 ± 2.16 ml·kg·min^{-1}; p = 0.03). These results are displayed in **Table 5.1**.

Table 5.1. Participant characteristics

Characteristic	Active (n=3)	Inactive (n=3)	P-value
Age (years)	24.67 ± 3.09	26.0 ± 4.32	0.3703
BMI (kg·m^2)	23.97 ± 0.78	22.3 ± 1.84	0.1515
MVPA (minutes·week)	470 ± 61	98 ± 20	0.0006***
$\dot{V}O_{2peak}$ (ml·kg·min^{-1})	43.23 ± 3.56	35.36 ± 2.16	0.0265*

*Values shown are mean ± SD, * p < 0.05, ***p < 0.001*

5.3.2 Serum Preconditioning Effects on Endothelial Cell Morphology After Exposure to FEC-T Drugs

To investigate the impact of habitual physical activity on chemotherapy-induced endothelial cell toxicity, a serological preconditioning cell culture model assay was performed, where endothelial cells were preconditioned with serum from physically active and inactive individuals prior to chemotherapy drug exposure. With active serum preconditioning, endothelial cells appear to be protected from the effect of FEC-T exposure, with fewer clumping of cells observed in the habitually active serum preconditioning compared to inactive serum preconditioning (**Figure 5.2**).

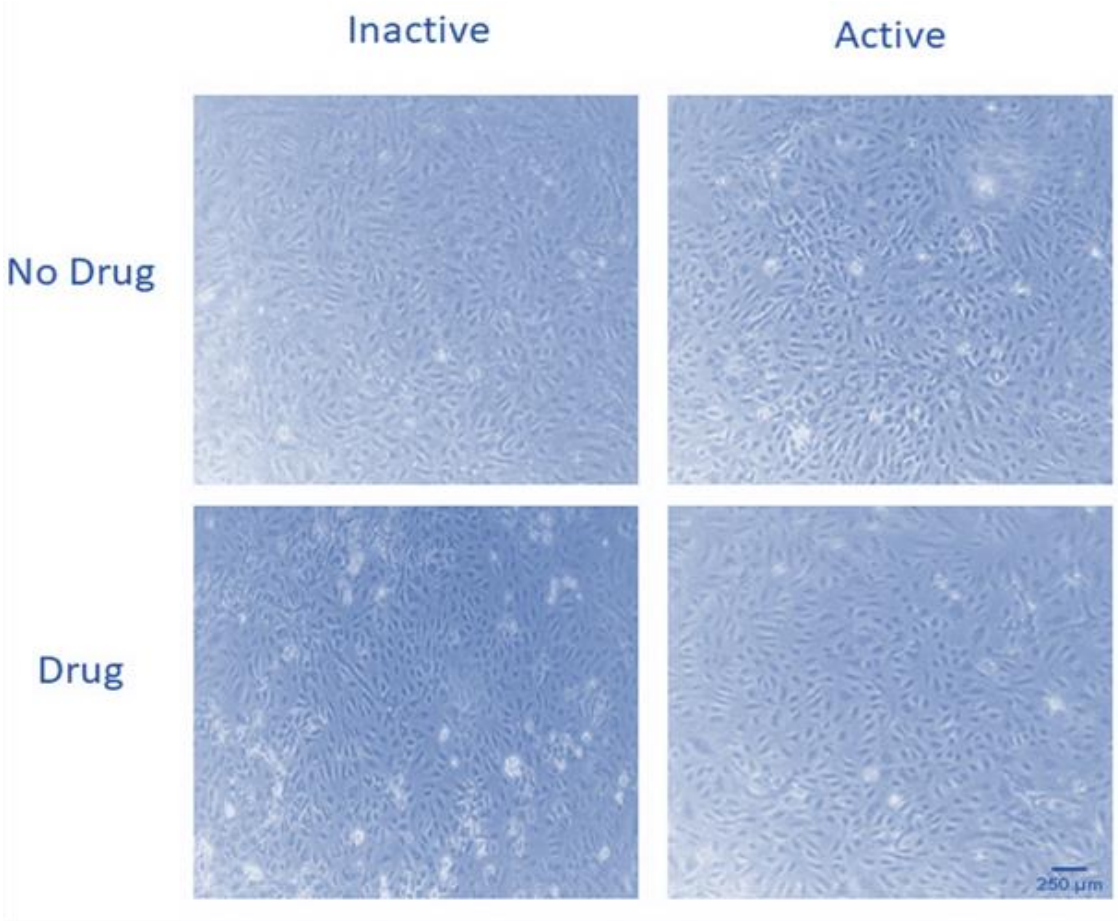

Figure 5.2. Representative images of endothelial cell monolayers with and without exposure to chemotherapy drugs with inactive and active serum preconditioning. *Scale bar 250µm.*

5.3.3 Serum Preconditioning Effects on Endothelial Cell Viability, Annexin-V, and Cleaved Caspase-3 Expression After Exposure to FEC-T Drugs

One-way ANOVAs revealed statistically significant differences between physically active and inactive serum preconditioning on the effects of 5-FU exposure on HUVECs (**Figure 5.3**). At 3- and 12-hours post exposure to 5-FU there was a significantly higher number of viable cells (3 hours active: 96.51 ± 1.73% vs inactive: 87.99 ± 2.94%, $p = 0.033$; 12-hours active: 83.50 ± 7.49% vs inactive: 66.13 ± 2.87%, $p = 0.048$) (**Figure 5.3A and D)**, and at 3-hours there was lower expression of phosphatidylserine (Annexin V binding) in active (compared to inactive (56.22 ± 6.78% vs. 74.23 ± 4.24%, $p = 0.044$) (**Figure 5.3C**). Cleaved caspase-3 expression did not show any between-group differences at either time point.

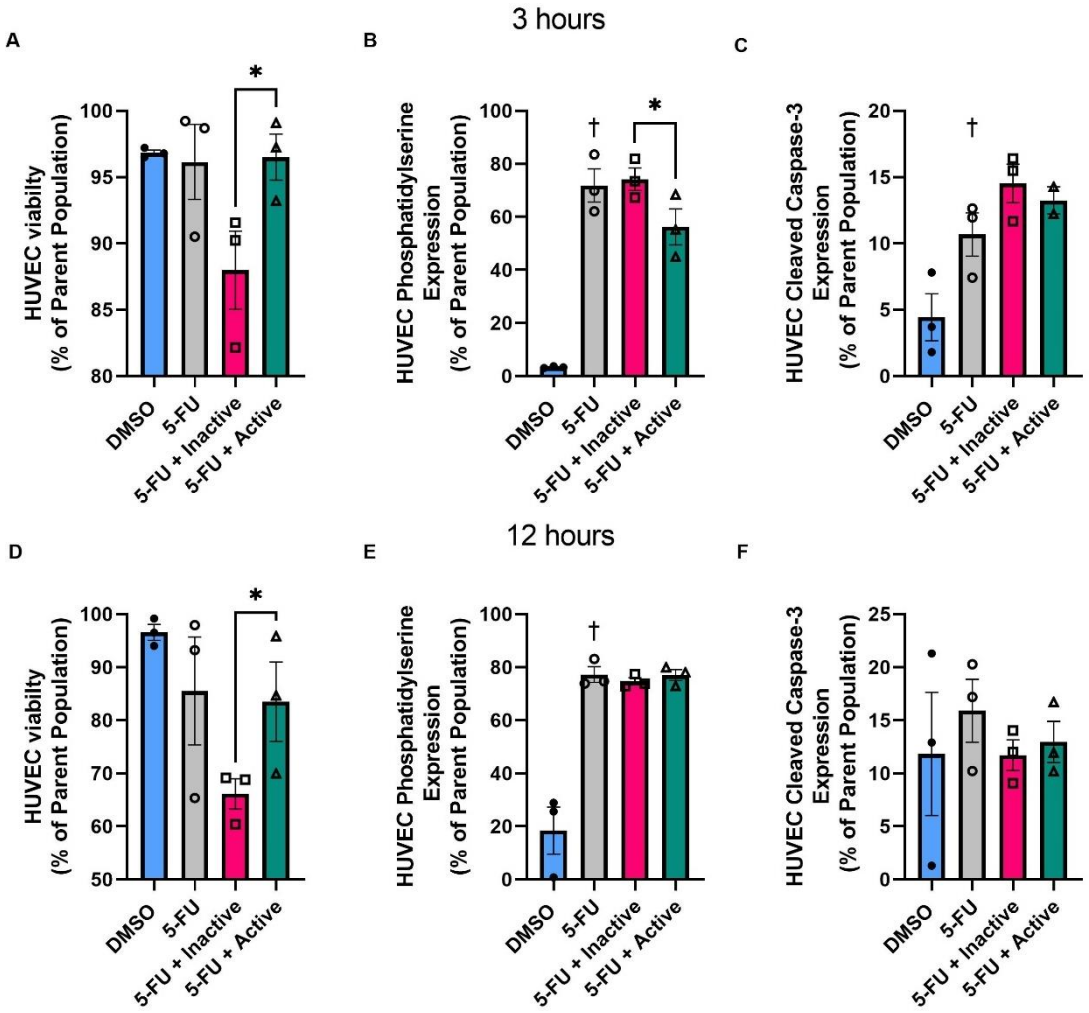

Figure 5.3. Comparison of inactive and active serum preconditioning on the effects of 1.5µM 5-fluorouracil on HUVEC cell viability (A, D), phosphatidylserine (B, E) and cleaved caspase-3 (C, F) expression at 3- (A, B, C) and 12-hours (D, E, F), as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p < 0.05, † significant compared to DMSO, Ψ significant compared to drug only condition.*

There were also some differences between habitual activity status for epirubicin exposure, with a statistically significant reduction in cleaved caspase-3 (active: 2.15 ± 0.17% vs inactive: 7.56 ± 1.11%, *p*=0.004) and annexin V binding to phosphatidylserine (active: 3.06 ± 0.26% vs inactive: 5.95 ± 1.24%, *p*=0.042) at 12-hours post-exposure (**Figure 5.4**).

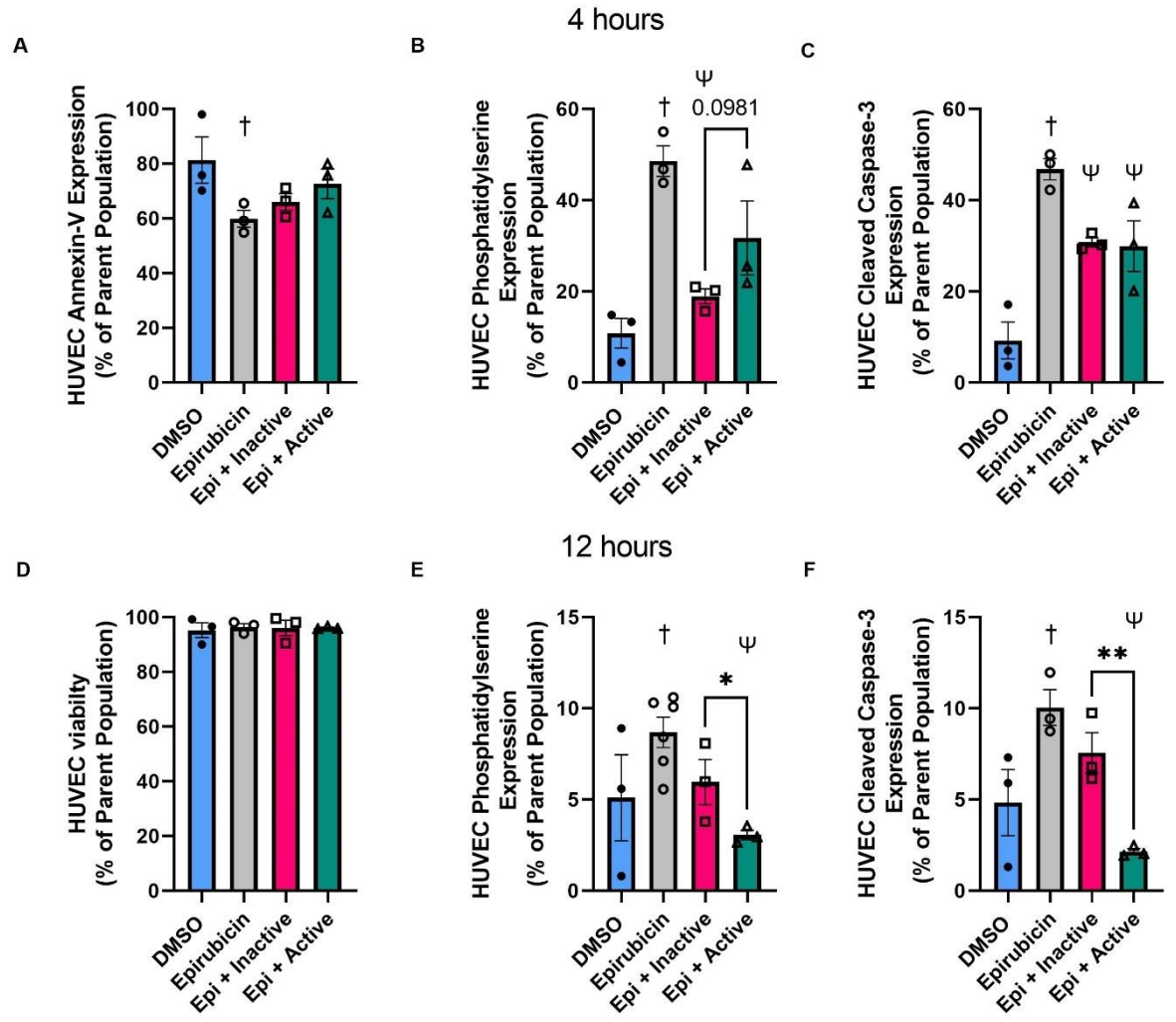

Figure 5.4. Comparison of inactive and active serum preconditioning on the effects of 0.006µM epirubicin on HUVEC cell viability (A, D), phosphatidylserine (B, E) and cleaved caspase-3 (C, F) expression at 4- (A, B, C) and 12-hours (D, E, F), as determined by ANOVA. *Data shown are mean ± SEM (n=5). *p < 0.05, ** p < 0.01, † significant compared to DMSO, Ψ significant compared to drug only condition.*

For cyclophosphamide, there were no significant between-group differences for viability, cleaved caspase-3, or phosphatidylserine expression with active compared to inactive at any time point (**Figure 5.5**).

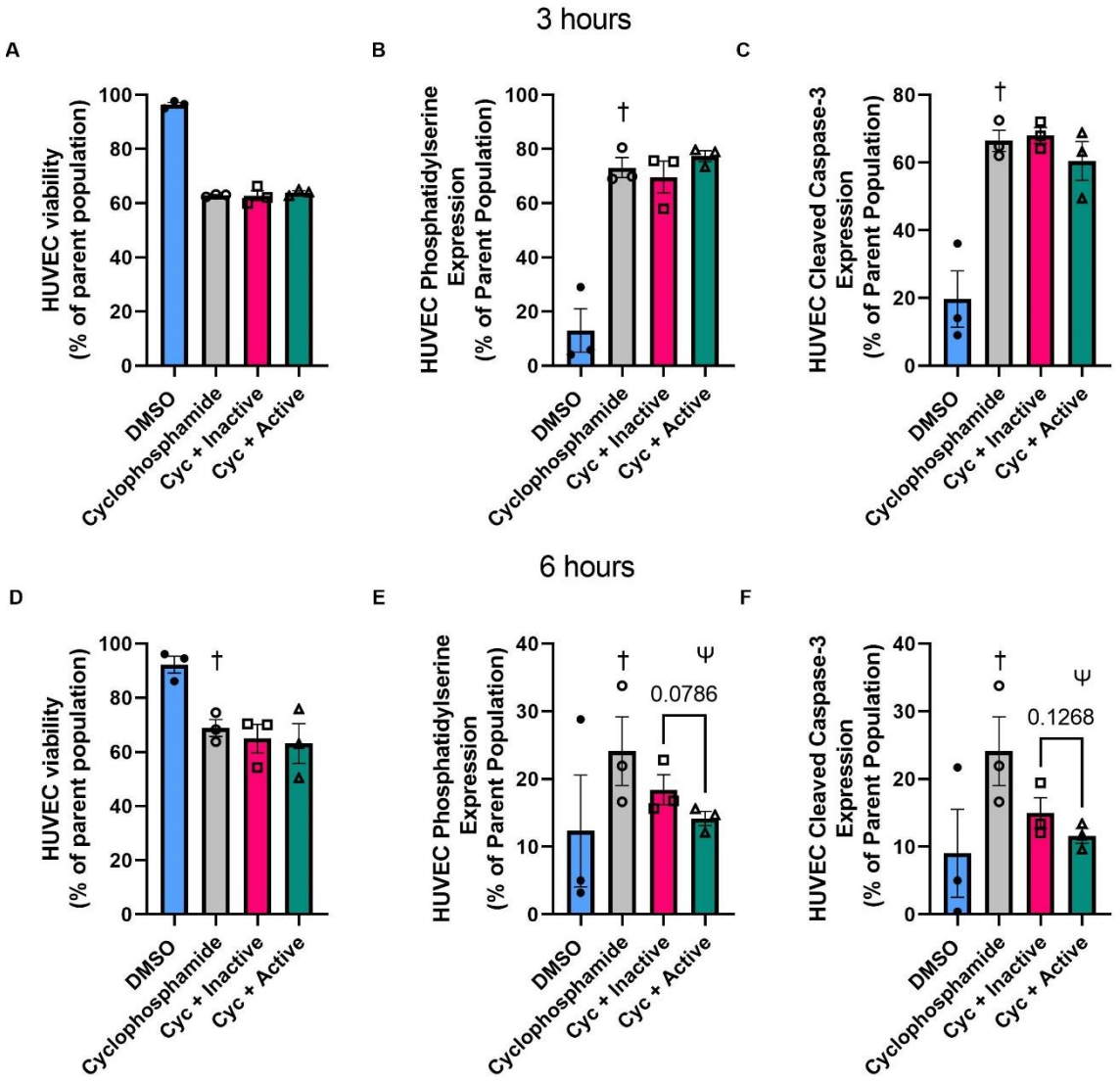

Figure 5.5. Comparison of inactive and active serum preconditioning on the effects of 38µM cyclophosphamide on HUVEC cell viability (A, D), phosphatidylserine (B, E) and cleaved caspase-3 (C, F) expression at 3- (A, B, C) and 6-hours (D, E, F), as determined by ANOVA. *Data shown are mean ± SEM (n=3). † significant compared to DMSO, Ψ significant compared to drug only condition.*

For HUVECs exposed to the combined FEC drugs, there were no significant between-group differences for viability, cleaved caspase-3, or phosphatidylserine expression with active compared to inactive at any time point (**Figure 5.6**). Likewise, there were no statistical differences between active and inactive serum preconditioning in HCAECs exposed to FEC for cleaved caspase-3 or phosphatidylserine expression (**Figure 5.7**).

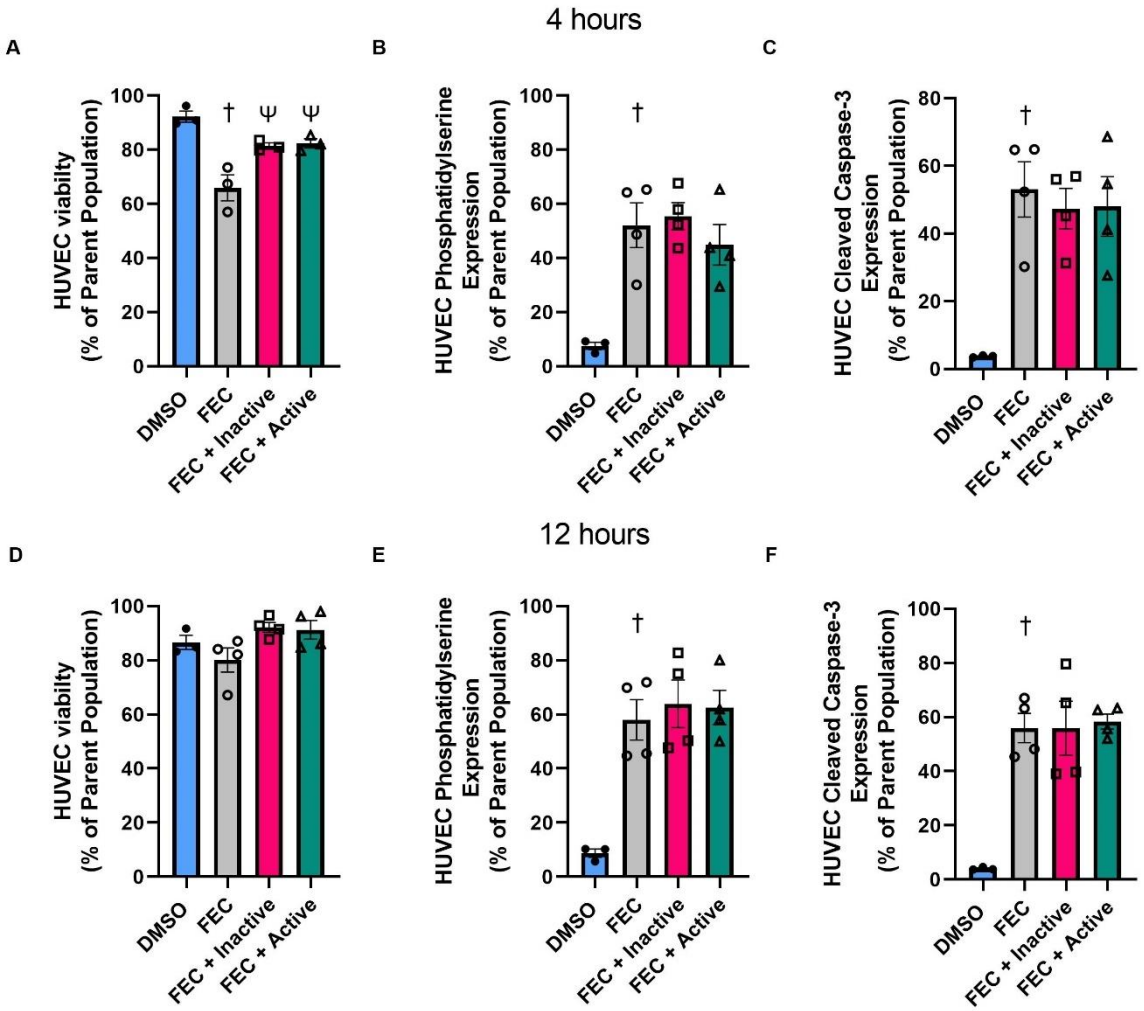

Figure 5.6. Comparison of inactive and active serum preconditioning on the effects of FEC (5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) on HUVEC cell viability (A, D), phosphatidylserine (B, E) and cleaved caspase-3 (C, F) expression at 4- (A-C) and 12-hours (D-F), as determined by ANOVA. *Data shown are mean ± SEM (n=4). † significant compared to DMSO, Ψ significant compared to drug only condition.*

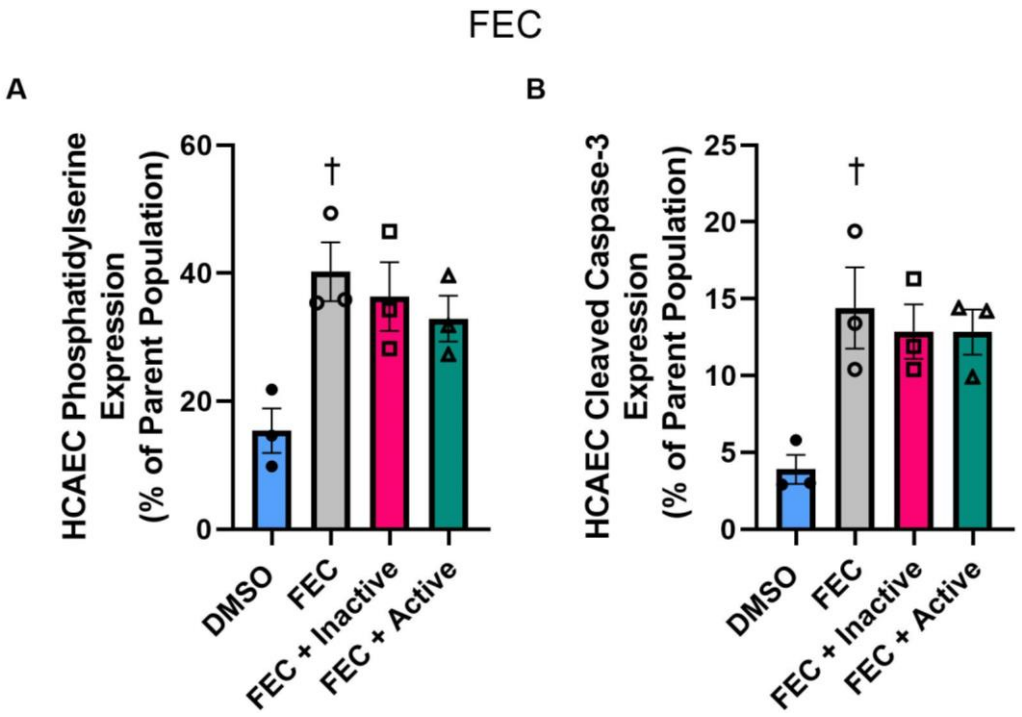

Figure 5.7. Comparison of inactive and active serum preconditioning on the effects of FEC (5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) on HCAEC phosphatidylserine (A) and cleaved caspase-3 (B) expression at 4-hours post-exposure, as determined by ANOVA. *Data shown are mean ± SEM (n=3). † Significant compared to DMSO.*

For HUVECs exposed to docetaxel, there were no significant between-group differences for viability, cleaved caspase-3, or phosphatidylserine expression with active compared to inactive at any time point (**Figure 5.8**). However, HCAEC experiments revealed a significant between-group difference for docetaxel, with significantly lower phosphatidylserine expression in active compared to inactive serum preconditioned HCAECs (**Figure 5.9A**).

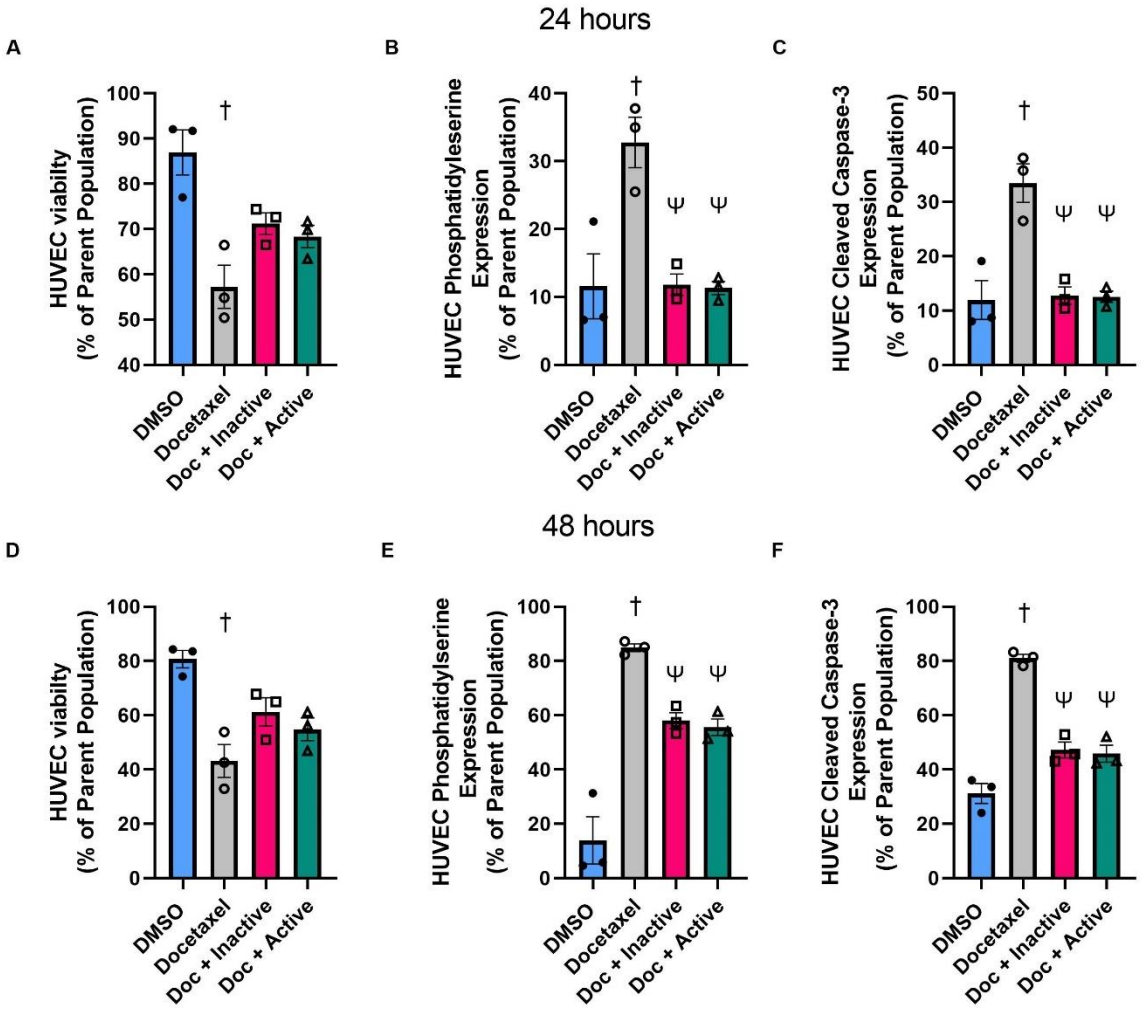

Figure 5.8. Comparison of inactive and active serum preconditioning on the effects of 6µM docetaxel on HUVEC cell viability (A, D), phosphatidylserine (B, E) and cleaved caspase-3 (C, F) expression at 24- (A-C) and 48-hours (D-F), as determined by ANOVA. *Data shown are mean ± SEM (n=3). † Significant compared to DMSO, Ψ significant compared to drug only condition.*

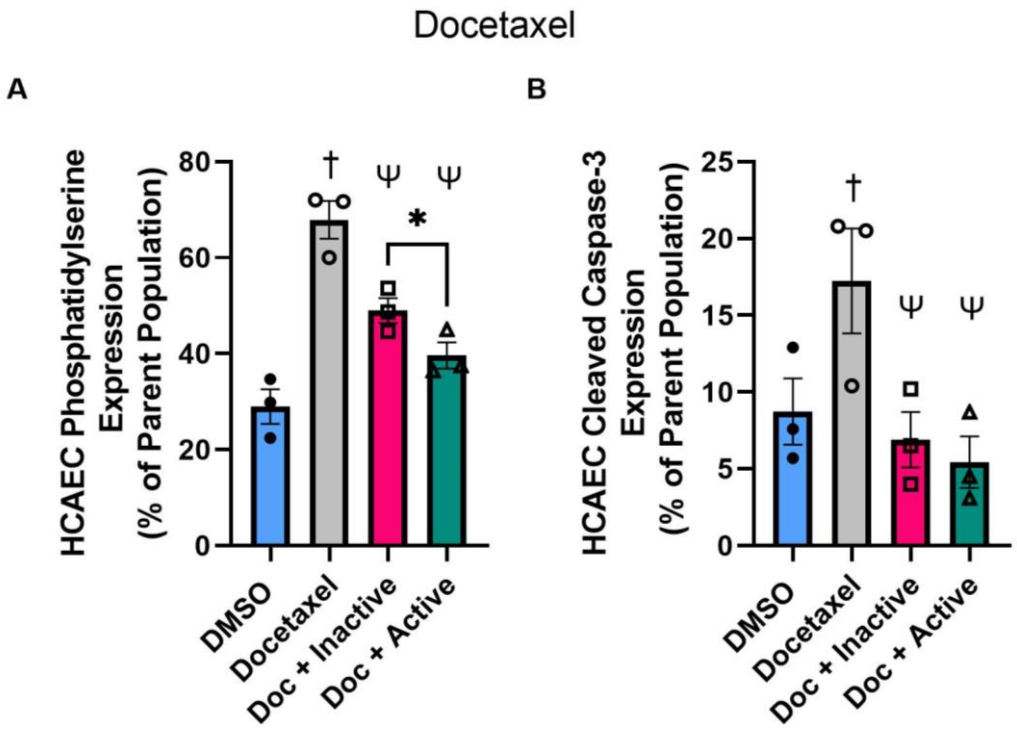

Figure 5.9. Comparison of inactive and active serum preconditioning on the effects of 6µM docetaxel on HCAEC phosphatidylserine (A) and cleaved caspase-3 (B) expression at 48-hours post-exposure, as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p < 0.05. † Significant compared to DMSO, Ψ significant compared to drug only condition.*

5.3.4 Serum Preconditioning Effects on Endothelial Cell eNOS and Phospho-eNOS Expression After Exposure to FEC-T Drugs

For HUVECs, eNOS expression and phosphorylation state did not show any statistically significant differences for habitual activity status on exposure to FEC drug combination (**Figure 5.10**). All data are presented in supplementary figure (**Appendix 13**).

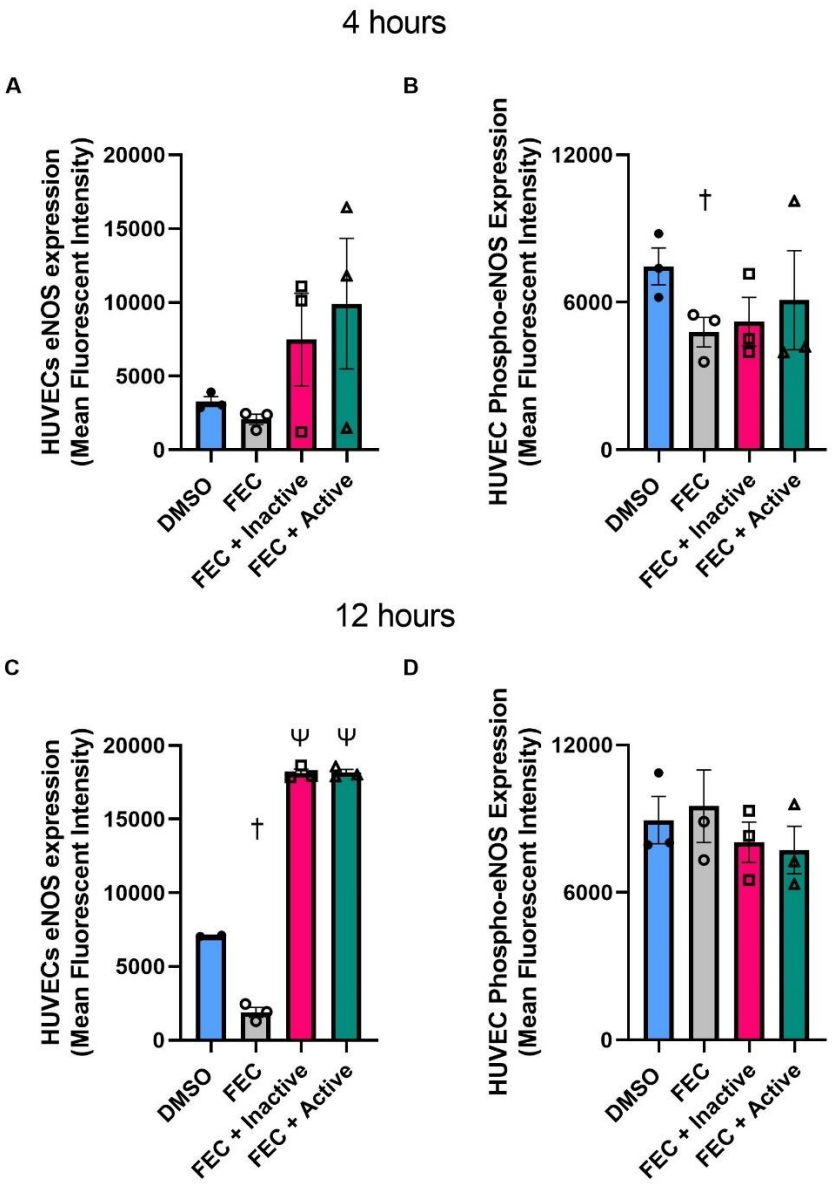

Figure 5.10. Comparison of inactive and active serum preconditioning on the effects of combined FEC (5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) chemotherapy on HUVEC eNOS expression at 4- (A) and 12-hours (C), and eNOS activation state measured by expression of phosphorylation state of eNOS at 4- (B) and 12-hours (D), as determined by ANOVA. *Data shown are mean ± SEM (n=3). † Significant compared to DMSO, Ψ significant compared to drug only condition.*

Similarly, there was no between-group difference for HCAEC eNOS expression with FEC treatment (**Figure 5.11A**). However, active serum preconditioning resulted in higher eNOS expression when compared to inactive serum in the docetaxel experiments (inactive: 2527 ± 140 vs active: 3032 ± 149, p = 0.034) (**Figure 5.11B**).

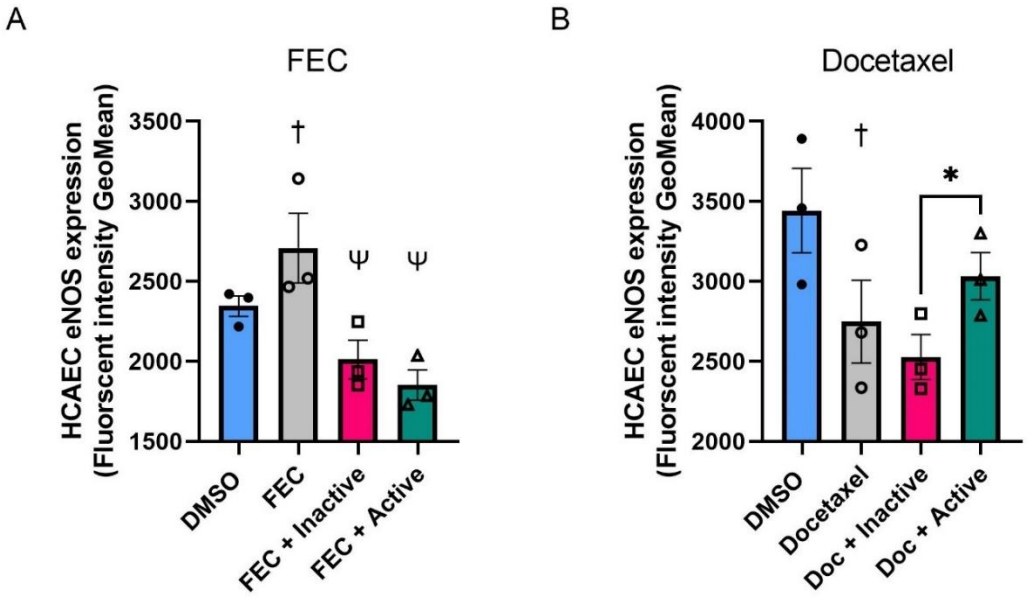

Figure 5.11. Comparison of inactive and active serum preconditioning on the effects of FEC (A: 5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) and 6µM docetaxel (B) on HCAEC eNOS expression at 4- and 48-hours, respectively, as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p < 0.05, † significant compared to DMSO, Ψ significant compared to drug only condition.*

5.3.5 Serum Preconditioning Effects on Endothelial Cell Adhesion Molecule Expression After Exposure to FEC-T Drugs

HCAECs adhesion molecule expression analysis produced significant differences between active and inactive serum groups, with significantly higher ICAM-1 expression in the active group (399 ± 35 MFI vs 710 ± 53 MFI, p = 0.004, **Figure 5.12A**) with FEC exposure. Adhesion molecule expression analysis for docetaxel experiments also revealed significantly higher ICAM-1 in active vs inactive groups (396 ± 37 MFI vs 693 ± 56 MFI, p = 0.006, **Figure 5.12B**).

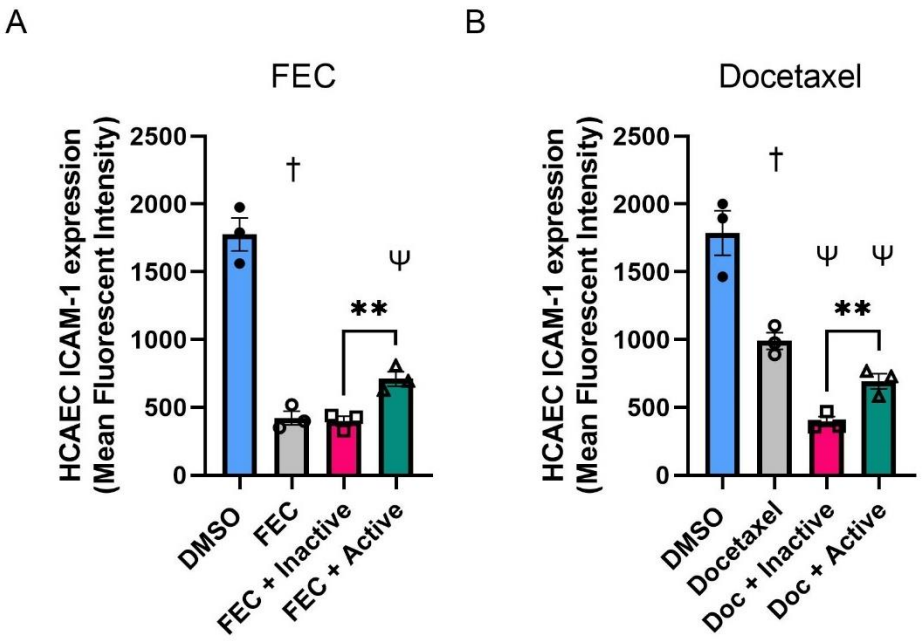

Figure 5.12. Comparison of inactive and active serum preconditioning on the effects of FEC (A: 5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) and 6.00µM docetaxel (B) on HCAEC ICAM-1 expression at 4- and 48-hours, respectively, as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p<0.05, † significant compared to DMSO, Ψ significant compared to drug only condition.*

5.3.6 The Effects of Serum Preconditioning on HUVEC Wound Healing After Exposure to FEC-T Drugs

Representative images of HUVEC and HCAEC wound healing comparing active vs inactive serum preconditioning are presented in **Figure 5.13** and comparison of gap closure over time are presented in **Figure 5.14 and 5.16**. For ease of analysis and interpretation of results, wounds which increased in size over time were given a value of 0.00µm/hr for rate of gap closure. The effects of preconditioning HUVECs with serum did not differ with physical activity levels with exposure to 5-FU, epirubicin, or cyclophosphamide drugs individually, but did reach significance in the combined FEC treatments, with a significantly faster rate of gap closure, as measured by $V_{migration}$ (p = 0.009) and $T_{1/2gap}$ (p = 0.003), with habitually active serum preconditioning (**Appendix 14**). There was significantly lower AUC for active vs inactive serum preconditioning in the docetaxel analysis only (**Figure 5.15E**). Analysis of HCAEC wound healing assay revealed between-group differences for $V_{migration}$ and AUC in the docetaxel condition only (p = 0.037 and 0.01, respectively). Further analysis of $T_{1/2gap}$ revealed significant between group difference in favour of the physically active group for 5-FU (p = 0.022). However, there was no between-group differences for any of the other chemotherapy combinations for $V_{migration}$, area under the curve or $T_{1/2gap}$. Full results are displayed in **Appendix 16**.

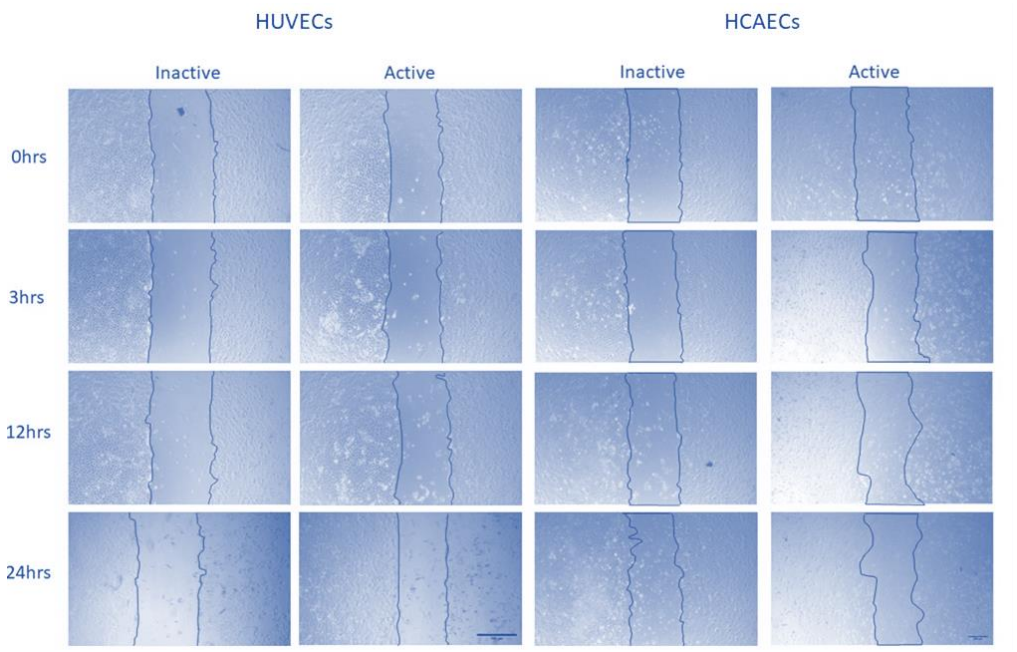

Figure 5.13. Representative images of HUVEC and HCAEC gap closure over time comparing inactive and active serum preconditioning. *Images are X4 magnification. Scale bar is 250µm.*

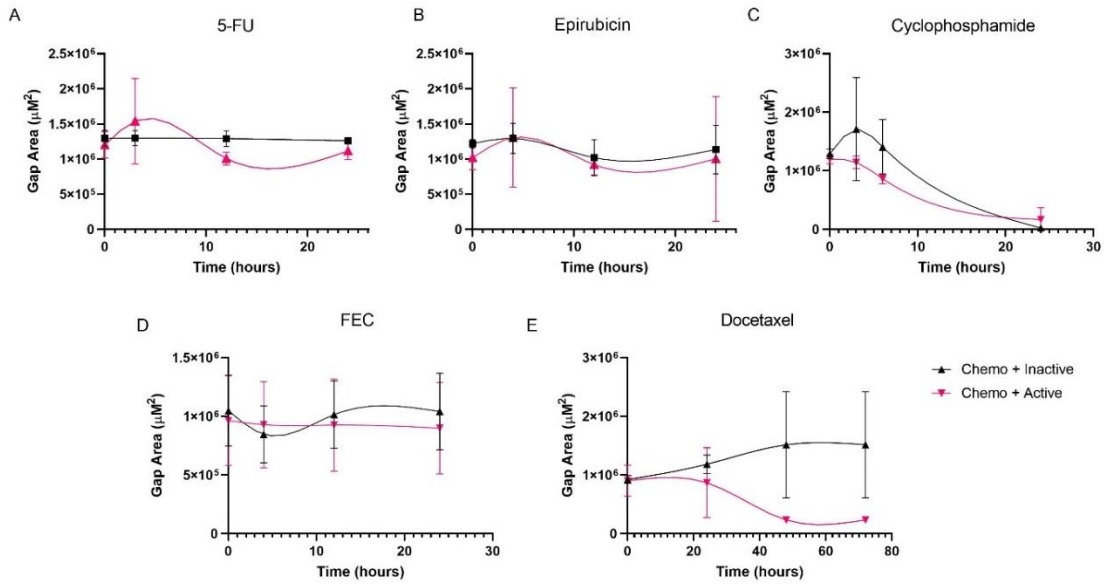

Figure 5.14. HUVEC wound closure (Gap area (μM^2)) over time (hours) with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), docetaxel (D), and combined FEC treatments (E) comparing inactive and active serum preconditioning. *Data shown are mean ± SEM (n=3).*

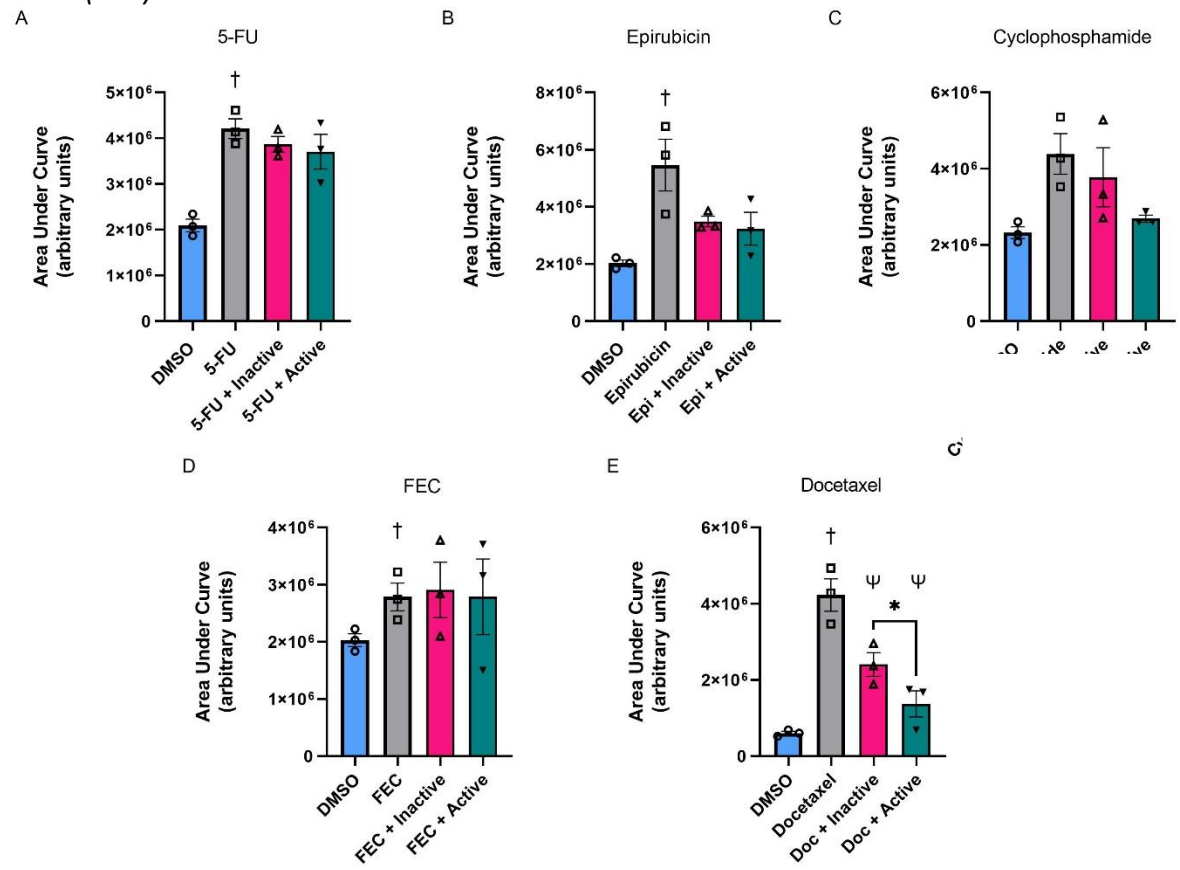

Figure 5.15. Area under the HUVEC gap closure curve with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), docetaxel (D), and combined FEC treatments (E) with no serum, inactive serum, active serum, displaying media only and DMSO no drug vehicle controls, as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p<0.05, † significant compared to DMSO, Ψ significant compared to drug only condition.*

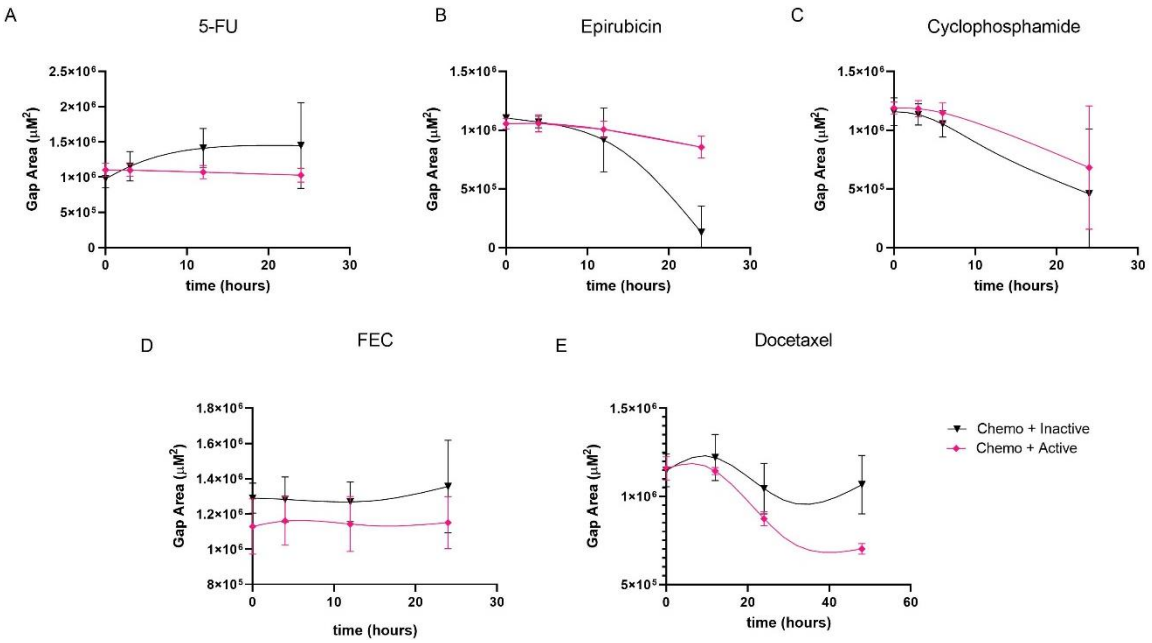

Figure 5.16. HCAEC wound closure (Gap area (μM^2)) over time (hours) with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), docetaxel (D), and combined FEC treatments (E) comparing inactive and active serum preconditioning. *Data are presented as Mean ± SEM (n=3).*

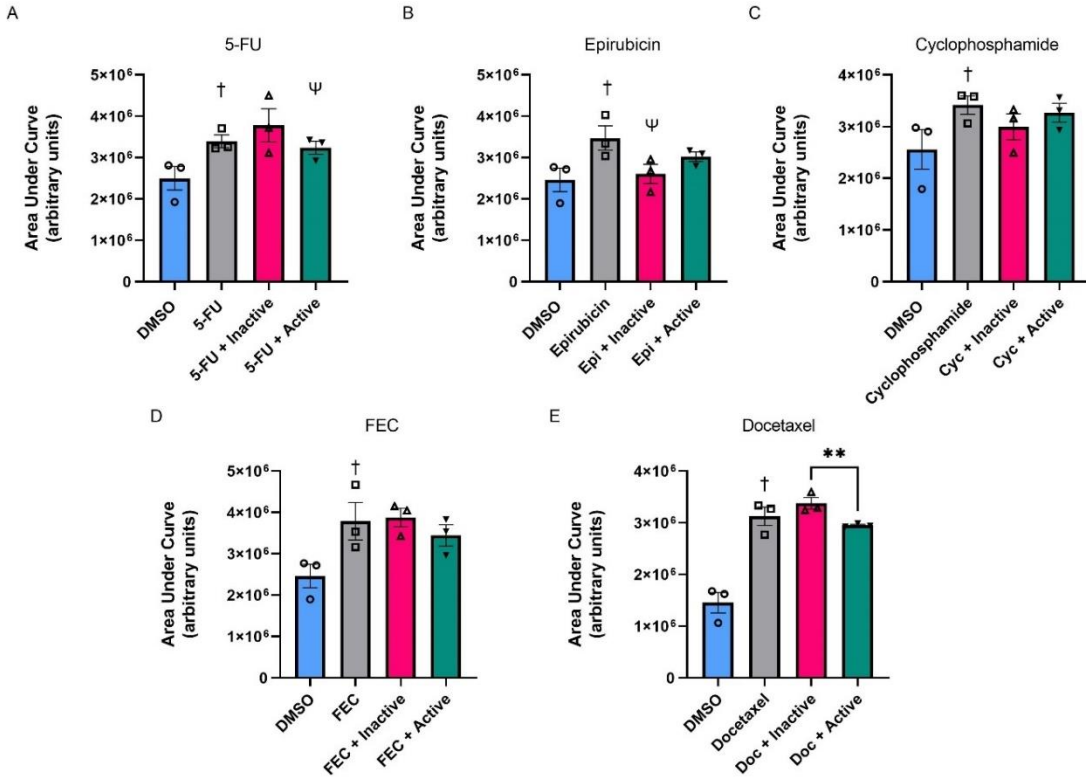

Figure 5.17. Area under the HCAEC gap closure curve with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), docetaxel (D), and combined FEC treatments (E) with no serum, inactive serum, active serum, displaying media only and DMSO no drug vehicle controls, as determined by ANOVA. *Data shown are mean ± SEM (n=3). **p<0.01, † significant compared to DMSO, Ψ significant compared to drug only condition*

5.4 Discussion

The aim of this study was to determine if there was a protective effect of habitual physical activity on vascular toxicity of chemotherapy using an *ex vivo* serum preconditioning approach. The current findings show that active serum has a protective effect on health of HUVECs when exposed to 5-FU and epirubicin but has no real effect on HUVEC health when exposed to cyclophosphamide, docetaxel or the combined FEC treatment, as measured by cell viability, phosphatidylserine, and cleaved caspase-3 expression, eNOS expression and phosphorylation. However, active serum preconditioning does have protective effects on HUVEC wound repair when exposed to both combined FEC and docetaxel. For HCAECs exposed to FEC, adhesion molecule expression was higher in active vs inactive preconditioning under stimulated conditions but there was no between-group difference for any other outcome measures. However, for docetaxel exposure there were protective effects of active serum as shown by decreased phosphatidylserine expression, increased eNOS expression, and increased adhesion molecule expression. Exercise vasculo-oncology is a relatively new field of research and therefore, there are currently no other known *in vitro* studies investigating a protective effect of exercise on vascular toxicity of chemotherapy using an *ex vivo* serum preconditioning approach. Therefore, as the current study is very novel, there are no directly comparable findings within the literature. However, other *ex vivo* models validate the method of serum preconditioning in exercise oncology research (Devin et al., 2019).

5.4.1 Serum Preconditioning from Active vs Inactive Individuals on Effects of FEC-T Drugs on Endothelial Cell Viability

With regards to serum preconditioning protection, it can be concluded that serum from habitually active females has a significant protective effect on endothelial cells 3-hours after exposure to 5-FU (**Figure 5.3**), with 10% higher cell viability and 23% lower phosphatidylserine expression (determined by Annexin V binding) in comparison to inactive control serum ($p < 0.05$ for both). At 12-hours post-exposure, the number of viable endothelial cells was 26% higher in the physically active group, strengthening evidence that active serum has a protective effect against 5-FU exposure *in vitro*. Furthermore, active serum had significant protection against epirubicin exposure, with a 71% reduction in

cleaved caspase-3 and a 50% reduction in phosphatidylserine expression in HUVECs at 12 hours post-exposure, compared to inactive serum preconditioning (**Figure 5.4E and F**), suggesting a protective effect of habitual physical activity against endothelial toxicity of epirubicin treatment. In HCAECs, there was a protective effect of active serum compared to inactive serum on docetaxel exposure, with 20% lower phosphatidylserine expression (**Figure 5.9A**), again suggesting a reduction in the early phase of endothelial apoptosis. Despite these significant findings, it must be noted that there was no between-group difference for viability, cleaved caspase-3, or phosphatidylserine expression with combined FEC drug exposure in both HUVECs and HCAECs. This may be due to the combined toxicity being too severe to overcome with serum preconditioning. However, this was also the case for individual drugs cyclophosphamide and docetaxel in HUVECs. Perhaps, the differing mechanisms of toxicity are important when alleviating toxicity with serological preconditioning. Regardless, our findings that active serum protected against toxicity of 5-FU and epirubicin in HUVECs and against docetaxel in HCAECs, are hopeful for the field of exercise cardio-oncology.

This endothelial protection is echoed by an *ex vivo* study investigating the protective effects of long-term aerobic exercise against oxidative stress by preconditioning human endothelial cells to sera from aerobic athletes, using anaerobic athletes' serum as controls (Conti et al., 2012b). Endothelial cell viability was higher in cultures supplemented with serum of aerobic athletes before exposure to oxidative stress, likely due to increased antioxidants in the serum (Conti et al., 2012b). Within cells, the antioxidants, superoxide dismutase and glutathione peroxidase respectively dismutate superoxide radicals and breakdown hydroperoxides to non-harmful substrates in order to reduce oxidative stress (Ighodaro & Akinloye, 2018). Lifelong physically active individuals have higher circulating levels of superoxide dismutase and glutathione peroxidase than their sedentary counterparts (Bouzid et al., 2018). Higher levels of these antioxidants results in reduced oxidative stress (Bouzid et al., 2018), preventing subsequent mitochondrial damage (Bouzid et al., 2018), and induction of the intrinsic apoptotic pathway (Elmore, 2007). This is interesting as epirubicin and docetaxel both work via oxidative stress pathways (Florescu et al., 2013; Stěrba et al., 2013). Hence, attenuation of oxidative stress-induced damage via enhanced serum antioxidants could explain the protective effects of active serum found in the current study

(**Figure 5.4E, F and 5.9B**). Further explanations for these findings can be taken from non-exercise studies of chemotherapy toxicity on cardiac cells. It has been shown that addition of HDL protects against doxorubicin-induced cardiomyocyte apoptosis via activation of anti-apoptotic pathways (Durham, Chathely, & Trigatti, 2018; Frias et al., 2010). As physically active individuals have increased circulating levels of HDL (Kodama et al., 2007), this is also a plausible explanation for the alleviation of chemotherapy-induced endothelial cell apoptosis with active serum preconditioning observed within the current study (**Figure 5.4E, F and 5.9A**).

Vascular protection of physical activity against chemotherapy toxicity is further paralleled in human studies as BC patients who participated in ≈18 MET-h·wk^{-1} compared with <2.5 MET-h·wk^{-1} before and during treatment was associated with a 31% and 47% lower risk of CAD and any CVD event death, respectively, after a median follow-up of 12.7 years (Palomo et al., 2017). Similarly, cancer patients with high and intermediate CRF had adjusted HR of 0.40 and 0.41 respectively, for cardiovascular mortality when compared to those with low CRF (Groarke et al., 2020). However, the underlying mechanisms of this protective effect remain largely unknown (J. M. Scott, Nilsen, Gupta, & Jones, 2018b). Now, we have shown for the first time that protective effects of physical activity against chemotherapy may be, in part, explained by enhanced endothelial cell survival.

5.4.2 Serum Preconditioning from Active vs Inactive Individuals on Effect of FEC-T Drugs on Endothelial Nitric Oxide Synthase Expression

With regards to exercise training status, there was a protective effect of active serum preconditioning on eNOS expression with exposure to docetaxel in HCAECs (**Figure 5.11B**). However, there were no statistically significant differences in eNOS expression or activation between active and inactive groups with exposure to FEC drug combination in HUVECs or HCAECs (**Figure 5.10 and 5.11A**). These findings are similar to those for viability of endothelial cells as active serum preconditioning has a protective effect with docetaxel but not with the combined FEC treatment. This may be due to NO production inhibition in apoptotic endothelial cells (Sato et al., 1993). The different findings between drug conditions may be due to the combined toxicity of FEC drugs or due to the differing mechanisms of action. Docetaxel induces oxidative stress (Florescu et al., 2013) which may

be counteracted via enhanced serum anti-oxidants in physically active individuals (Bouzid et al., 2018; Conti et al., 2012b), protecting against the oxidative stress-induced reduction in eNOS with docetaxel (**Figure 5.11B**). Epirubicin also induces oxidative stress (Stěrba et al., 2013). However, when coupled with cyclophosphamide and 5-FU which induce DNA and RNA damage (Chighizola et al., 2011; Longley et al., 2003), these mechanisms may override the protective effects of serum antioxidants. Hence, differences in findings between FEC and docetaxel exposure raise further questions for future research into physical activity protection against chemotherapy-induced endothelial dysfunction.

Interestingly, an *ex vivo* study of long-term physical activity found higher NO production in endothelial cells conditioned with aerobic serum, both at baseline and after oxidative stress exposure, suggesting that endothelial protection could, at least partly, be due to higher NO bioavailability with aerobic exercise serum (Conti et al., 2012b). Consistencies between this study and the current study strengthen our conclusions that physical activity protects against endothelial eNOS reduction with docetaxel exposure (**Figure 5.11B**).

These findings relate to *in human* studies of vascular health and function. In healthy non-cancer individuals, physical activity was directly associated with endothelial function as measured by FMD suggesting that people who participate in more physical activity have higher NO bioavailability (Pahkala et al., 2011). Physical activity also inversely associated with intima media thickness suggesting protective effects of increased NO content against atherosclerosis plaque development (Pahkala et al., 2011). There are no observational studies of physical activity and endothelial function in BC patients. However, these findings are still relevant as physical activity may precondition the circulatory system to an anti-thrombotic state before initiation of chemotherapy. This may be responsible for the protective effects of active vs inactive serum on eNOS content when exposed to docetaxel (**Figure 5.11B**). This could have physiological relevance as reduced NO bioavailability causes plaque development leading to CV events (Benjamin et al., 2004) which are prevalent in BC survivors (Patnaik et al., 2011b).

5.4.3 Serum Preconditioning from Active vs Inactive Individuals on Effects of FEC-T Drugs on Endothelial Cell Adhesion Molecule Expression

Chemotherapy exposure (no serum) downregulated ICAM-1 expression **(Figure 5.12)**. With active serum preconditioning, there was significantly higher ICAM-1 expression in both FEC, and docetaxel exposed endothelial cells when compared to inactive serum preconditioning (**Figure 5.12A&B**). The increase in ICAM-1 is possibly the active serum preconditioning restoring normal homeostasis of the endothelial cell more similar to baseline expression, reversing the downregulatory effects of docetaxel exposure on ICAM-1. A potential explanation for this is the reduction in apoptosis also evidenced in this study (Figure). A reduction in endothelial cell apoptosis may well have reduced membrane blebbing and therefore, preserved surface expression of adhesion molecules (Navratil et al., 2001; Volanti et al., 2004). There are no other known *in vitro/ex vivo* studies of exercise serological preconditioning on chemotherapy toxicity on endothelial cells by which to compare these findings. Therefore, these results are novel and further investigation is required for confirmation and exploration of both consequence and mechanism.

A review of the effects of physical activity associations with adhesion molecule expression in humans (non-cancer) found that exercise elicited either no change or a decrease in soluble ICAM-1 (Palmefors, DuttaRoy, Rundqvist, & Börjesson, 2014b). Therefore, our findings are in contrast to the literature. The main mediator of exercise-induced regulation of adhesion molecules has been determined to be shear stress (Tinken et al., 2010). Of course, the current study did not utilise shear stress and therefore, this may be where the differences in our results lie. However, the method of serum preconditioning is still relevant to this outcome measure as serological markers including HDL, which is upregulated in active populations (Kodama et al., 2007), also downregulates adhesion molecule expression (Kimura et al., 2006; Muñoz-Vega et al., 2018). Since no other *in vitro* study has investigated the effects of serological preconditioning of cells with habitually active serum on adhesion molecule expression, it is not possible to draw definite conclusions, but perhaps the addition of a chemotherapy agent reverses the protective effects of active serum preconditioning, but this is merely speculation. On the contrary, perhaps active serum preconditioning appears to upregulate adhesion molecule expression as the serum inhibits the potential for apoptotic blebbing and its effect on surface adhesion molecule expression

(Navratil et al., 2001; Volanti et al., 2004). Again, it is not possible to accept or reject this theory from the current data. As yet, there are no *in-human* studies investigating effects of physical activity alongside chemotherapy on adhesion molecule expression. This is the first ever study investigating effects of physical activity, chemotherapy, and endothelial adhesion molecules. Findings that active serum preconditioning increased adhesion molecule expression are novel and should be added to the literature with the caution that there is a demand for *in-human* chemotherapy studies to investigate this further. Alternatively, a shear stress culture model (Yin, Shanmugavelayudam, & Rubenstein, 2011) would also determine whether shear stress can modulate chemotherapy-induced toxicity in endothelial cells. This information is crucial to overcome the inconsistencies between findings in the current *in vitro* study and findings in the literature that exercise has been shown to reduce adhesion molecule expression in non-cancer populations. This will determine whether differences are because of lack of shear stress stimuli or because chemotherapy toxicity reverses the protective mechanisms of exercise serum.

5.4.4 Serum Preconditioning from Active vs Inactive Individuals on Effects of FEC-T Drugs on Endothelial Repair

FEC-T drugs significantly impaired endothelial wound repair (**Figure 5.14-17**). This study found that there were some improvements in endothelial wound repair with active serum preconditioning for both HUVECs and HCAECs. There was a significant between-group difference for HUVEC rate of gap closure upon exposure to the combined FEC chemotherapy (**Appendix 14**), and a significantly smaller AUC for docetaxel in both HUVECs and HCAECs (**Figure 5.15E & 5.17E**), in favour of active serum preconditioning. Similar results were found in a non-chemotherapy study whereby habitually active serum had a pro-proliferative effect on HUVECs, with serum from active women inducing significantly greater HUVEC migration and proliferation compared to serum from inactive women (Sapp et al., 2020). This is likely due to increased concentrations of growth factors, such as VEGF (Karakilic et al., 2021), in active serum as VEGF is heavily involved in directing migration in wound repair (Sapp et al., 2020). Furthermore, endothelial cell proliferation rates were higher in endothelial cell cultures supplemented with serum of aerobic athletes compared to anaerobic athletes, thought to be due to enhance NO content (Conti et al., 2012b). Chemotherapy-induced impairment of endothelial migration, as assessed by wound healing assay, has been shown

previously to be related to reduced NO production (which is dependent upon eNOS expression) (Gajalakshmi et al., 2013); as NO plays an important role in inducing migration within endothelial cells (Craige et al., 2011). In the current study, in HCAECs, active serum preserved both wound repair **(Figure 32E & 33E**) and eNOS content (**Figure 27B**) when exposed to docetaxel. Therefore, preserved NO production with docetaxel exposure may be the protective mechanism for migration-proliferation preservation observed in the HCAEC wound healing assay. There were no significant between-group differences for FEC drugs individually for both HUVECs and HCAECs. However, it is still a promising finding that serum from active females can counteract the inhibitory effect of combined FEC and docetaxel on endothelial cell ability to repair. As this is a treatment commonly given in early BC care, these findings have the potential for clinical importance as maintaining the ability of the endothelium to repair itself upon injury will help to reduce the risk of endothelial dysfunction and atherosclerosis, which are initiating steps for CVD.

5.4.5 Potentially Contributing Factors to Serological Attenuation of FEC-T Endothelial Cell Toxicity

Although VEGF (Karakilic et al., 2021; Sapp et al., 2020), antioxidants (Bouzid et al., 2018; Conti et al., 2012b), and HDL (Durham, Chathely, & Trigatti, 2018; Frias et al., 2010; Kodama et al., 2007) have been identified as potentially important contributors to the observed effects within this study, it must be noted that there are several other factors which are likely to also be involved. With physical activity, there are alterations in circulating inflammatory factors which may contribute to the observed effects. These include increased levels of anti-inflammatory factors such as interleukin-1 and -10 (Contrepois et al., 2020; Naylor et al. 2020) and reductions in pro-inflammatory cytokines such as TNF-α (Petersen & Pedersen, 2005). This is important for endothelial health and function as inflammatory signals activate endothelial cells and induce apoptosis (Joussen et al., 2009), with a chronically inflammatory environment resulting in atherosclerosis development (Insull, 2009; Sullivan et al., 2000).

As well as inflammatory mediators, metabolic factors, including glucose and insulin must also be considered as these are beneficially modulated with increased physical activity (Leskinen et al., 2013). This is likely to have a contributory effect on endothelial cells as a high glucose environment also induces endothelial cell apoptosis (Risso et al., 2001) and has

an effect on endothelial cell migration (Huang & Sheibani, 2008) and ICAM-1 expression (Altannavch et al., 2004).

It is also important to note that protection against chemotherapy-induced vascular endothelial toxicity may also be associated with reductions in oxidative stress which occur due to improved circulating antioxidant profiles and lipid signalling (Contrepois et al., 2020). Increased antioxidant profiles may directly counteract increased ROS with FEC-T chemotherapy (Clayton et al., 2020; Clayton, Hutton, et al., 2021; Stěrba et al., 2013). Improved lipid signalling may protect against endothelial cell death as HDL-cholesterol has been found to inhibit apoptotic signalling associated with doxorubicin exposure in cardiomyocytes (Durham, Chathely, Mak, et al., 2018; Durham et al., 2019).

Therefore, it is important for future investigations to consider investigation of metabolic markers, anti- and pro-inflammatory markers, and oxidative stress profiles to elucidate the whole picture of serological conditioning on endothelial cells exposed to FEC-T chemotherapy.

5.5 Conclusions and Implications

For the first time, we have shown that habitual physical activity can protect against 5-FU-, epirubicin-, and docetaxel-induced endothelial cell death and dysfunction *in vitro* using an *ex vivo* serum preconditioning approach. Additionally, active serum preconditioning protected against the usual docetaxel-induced eNOS downregulation and endothelial repair impairment. Physical activity in BC patients may reduce CV toxicity by similarly preconditioning the endothelium to an anti-apoptotic and anti-thrombotic state (Patnaik et al., 2011). However, only 27% of women with BC are physically active compared with 44% of non-cancer counterparts (Clark et al., 2017). To tackle this problem, the next steps are to determine whether an exercise intervention can elicit the same protection observed in habitually active females.

Chapter 6

Investigation of the potential for protective effects of acute exercise on the toxicity of FECT chemotherapy on HCAECs

6.1 Introduction

Cardiovascular toxicity is a burden for cancer patients, and it is responsible for dosage reductions and attenuations in chemotherapy regimens (Gavila et al., 2017). There is now emerging evidence that those who are physically active may have reduced risk of developing cardiovascular toxicity with chemotherapy (Nagy et al., 2017b). *Chapter 5* has added to this evidence with the finding that there is some protection of physical activity against FEC-T-induced endothelial apoptosis and wound repair disruption. However, for those who are not already 'active' before receiving chemotherapy, there is an increased risk of CV toxicity. Therefore, there is a need to determine whether these individuals can alleviate their risk of cardiotoxicity to improve chances of treatment completion. These individuals may benefit from an acute exercise intervention before treatment to limit toxicity by preconditioning the CVS to promote an anti-CVD profile (Newcomer et al., 2011; Palmefors et al., 2014a; Randers et al., 2013).

Acute exercise has the potential to protect the vasculature of cancer patients undergoing chemotherapy as, in CVD patients, there have been promising vascular benefits observed with acute exercise. A single moderate-intensity exercise session increased FMD by 1% at 10-minutes post-exercise in CVD patients (Bailey et al., 2018). Improved FMD after acute exercise may provide short-term benefits in those at risk of cardiovascular events and this has the potential to reduce the acute risk of endothelial dysfunction and cardiovascular events in BC patients undergoing chemotherapy. However, evidence from in-human chemotherapy studies is at an early stage, with only one study investigating acute exercise effects on subsequent cardiotoxicity. This involved 13 BC patients performing 30 minutes of vigorous-intensity exercise 24-hours prior to the first chemotherapy treatment, which resulted in decreased systemic vascular resistance, increased ejection fraction and systolic strain rate from pre- to post-chemotherapy compared with a non-exercise group (Kirkham et al., 2017). Relative to the control group, the exercise group had a 46% risk reduction of HF (Kirkham et al., 2017). Although these findings are promising, there is a clear need for further assessment of acute exercise effects and whether these can protect against toxicity, and the location of toxicity. No study has assessed the potentially protective effects of acute exercise before chemotherapy treatment on vascular toxicity. It is proposed that assessment of vascular protective effects of exercise against detriments of chemotherapy

are investigated as these often precede clinical cardiotoxicity and provide an avenue for early attenuation of cardiovascular harm (Verma et al., 2003).

Acute exercise increases circulating levels of anti-inflammatory factors IL-6, IL-1Ra and IL-10 (Petersen et al., 2011), inhibiting inflammation (Petersen et al., 2011). This is important for endothelial cells as inflammation is a stimulus for endothelial cell activation and apoptosis (Joussen et al., 2009). Furthermore, acute exercise reduces blood glucose concentrations (Lira et al., 2017), which may also reduce endothelial cell damage (Altannavch et al., 2004; Risso et al., 2001). It is important to note that acute-exercise induced alterations in blood-based factors are influenced by exercise intensity (Wilhelm, González-Alonso, Parris, & Rakobowchuk, 2016) and therefore, exercise protocols should be chosen carefully with this in mind.

The aim of this study is to determine whether there is a protective effect of a single exercise bout on endothelial cells exposed to chemotherapy for BC by using a combined human acute exercise intervention and *ex vivo* cell culture model, as used in the previous chapter. This will help to determine whether inactive individuals can alleviate their risk for vascular toxicity by performing a single exercise session before chemotherapy cycles. Currently, there are no specific exercise recommendations for an acute exercise session in those with BC (Campbell et al., 2019a). Therefore, the exercise session was chosen based on feasibility for those with BC as it was designed by MacMillan Cancer Charity exercise specialists to be performed in a home-based setting, improving reachability of the intervention, increasing likelihood of adherence (Ormel et al., 2018). It is hypothesised that endothelial cells preconditioned with post-acute exercise serum will have some protection against the toxicity of FEC-T drugs compared to pre-exercise serum.

6.2 Methodology

6.2.1 Participants

The 'inactive' (based on results from self-reported physical activity, measured by The International Physical Activity Questionnaire (<120 minutes/week MVPA)) participants from *Chapter 5* returned to complete an acute exercise study. Participants were healthy females, Caucasian, 18-35 years, BMI 18-24.9kg·m^{2,} currently taking oral contraceptives, and were excluded for presence of diabetes, neurological conditions, hypertension, or cardiovascular disease.

Ethical approval was granted from Edinburgh Napier University Research Integrity Committee and all participants provided written informed signed consent before participation in this study.

6.2.2 Acute Exercise Intervention

One week after the graded $\dot{V}O_{2peak}$ was carried out (as detailed in *Chapter 5*), the 'inactive' participants returned to the Edinburgh Napier University Sport and Exercise Science laboratory for a single acute exercise session. This consisted of following a MacMillan 'Get Active Feel Good' Move More Cancer Charity video comprised of aerobic movements and resistance exercises using light dumbbell weights (2kg) for 1 hour. Heart rate was recorded throughout using a chest strap monitor (H9 Beat, Polar, Finland). Prior to the exercise bout, and 10-minutes post-exercise, blood samples were obtained, as detailed in *Chapter 5*.

6.2.3 Endothelial Cell Serum Preconditioning

Unpooled coronary artery endothelial cells (HCAECs) (product code: 300-05A, Sigma-Aldrich, Gillingham, UK) were cultured in Meso-endothelial cell growth medium (MEGM; product code: 212-500, Sigma-Aldrich, Gillingham, UK), supplemented with 5% pooled pre- or post-acute exercise serum (Zitta et al., 2012), and incubated at 37^{o}C in 5% CO_2 for 24-hours.

6.2.4 Endothelial Cell Chemotherapy Exposure

Appropriate concentrations of 5-fluorouracil (product code: ab142387; Abcam, UK), epirubicin hydrochloride (product code: ab142100; Abcam, UK), cyclophosphamide (product code: ab141240, Abcam, UK) and docetaxel (product code: ab141248; Abcam, UK) were

added to the appropriate corresponding wells, as combined FEC and docetaxel alone. Drug concentrations were based on the serum concentration found in patients after exposure to each of these drugs (5-FU: 1.5µM (Reigner et al., 2003); epirubicin: 0.006µM (Danesi et al., 2002); cyclophosphamide: 38µM (Adams et al., 2014); and docetaxel: 6µM (Hurria et al., 2006)). Drugs were washed off at time-points corresponding to the literature findings when serum levels of each drug were diminished (Reigner et al., 2003);(Danesi et al., 2002); (Adams et al., 2014); (Hurria et al., 2006), and re-incubated at 37^{o}C in 5% CO_2 until the appropriate analysis time-points.

6.2.5 Sample Preparation for Analysis of Drug Effects

HCAECs were analysed at the most potent time-points, according to the most potent effects of the drugs found in *Chapter 4* (FEC: 4 hours; and docetaxel 48 hours). At the respective time-points, cells media was removed, and cells were washed with 200µl NaCl.

6.2.6 Flow Cytometry

Flow cytometry measurements were performed, as detailed in *Chapter 4*, using the following mAbs: anti-CD31-FITC (endothelial cell marker; product code: 303104; BioLegend, USA), anti-Annexin V-PerCP-Cy5.5 (product code: 561431; BD Biosciences, USA), anti-cleaved caspase-3-V450 (product code: 560627; BD Biosciences, USA) and anti-eNOS-PE (product code: 130-106-787; Miltenyi Biotec, Bergisch Gladbach, Germany). A separate panel was used for analysis of anti-ICAM-1 CD54-AlexaFluor 488 (product code: 322713, BioLegend, UK), as in *Chapter 4*. All samples were analysed using High Throughput System (HTS) on a 12-colour flow cytometer (FACS Celesta, BD Biosciences, USA).

6.2.7 Flow Cytometry Data Acquisition

Data were acquired using FACSDiva 6.0 Software (BD Bioscience, USA). Flow cytometer setup and calibration were performed using CS&T beads (BD Biosciences, USA). For sample acquisition, a minimum of 10,000 cell events were collected. Data were acquired using FACSDiva 6.0 Software (BD Bioscience, USA). Fluorescence compensation was performed as described in the Methodology and Materials chapter (*Chapter 3*).

6.2.8 Flow Cytometry Gating Process

Firstly, forward scatter and side scatter dot plots were created to determine the size and morphology of all events, allowing selection of the population of interest. A plot of forward scatter-area and -height was then created, gated on the population of interest. From this plot, singlets were selected. All subsequent plots were gated on singlets to remove any potential doublets from data analysis. FITC-CD31^{+} expression confirmed endothelial cell identity. Once gated on CD31^{+} cells, dot plots and histograms of annexin V-PerCP-Cy5.5, cleaved caspase-3-V450, and eNOS-PE could be visualised, and the data interpreted. ICAM-AlexaFluor 488 was analysed in the same way using a separate panel. Representative gating strategy is shown in **Figure 6.1**.

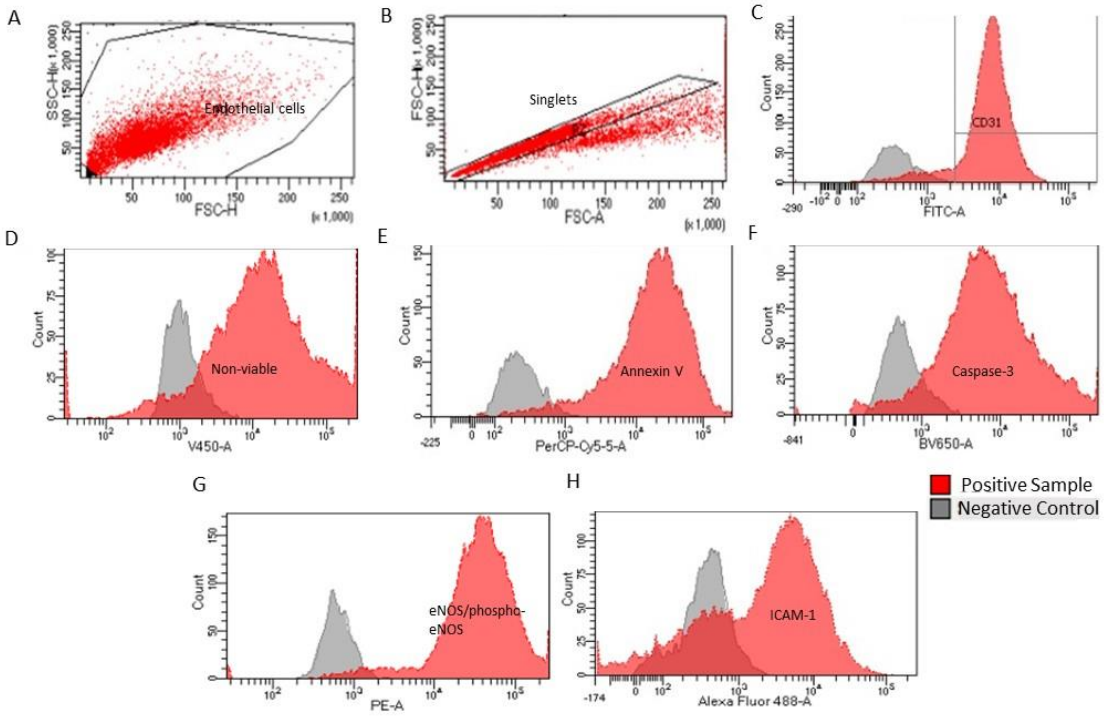

Figure 6.1. Representative gating strategy for HCAECs by morphology (A), singlets (B), and CD31 expression as displayed by FITC histogram (C). Histograms of Viability (D), Annexin V (E), caspase-3 (F), eNOS (G), and ICAM-1 (H) expression are displayed as a function of their respective lasers (PerCp-Cy.5, V450, PE, AlexaFluor488, respectively). *Histograms are displayed as GeoMean (Mean Fluorescent Intensity (MFI)).*

6.2.9 Wound Healing Assay

Endothelial cells were cultured in 24 well plates and treated with medium containing 5% pre- and post-exercise serum and incubated at 37ºC, 5% CO_2. After 24-hours of serum preconditioning, the endothelial monolayer was inflicted with a wound and analysed, as described in *Chapter 4*. Rate of gap closure was analysed using image analysis software (Image J 1.x, Java, USA). After measuring the gap area for each image, gap area was plotted as a function of time to derive the rate of wound closure. For analyses where the rate of wound closure was not different between groups, the $t_{1/2gap}$ value (the point at which the gap is half the original area) was also calculated, as in *Chapter 4.*

6.2.10 Statistical Analysis

To assess acute exercise-induced changes in participant characteristics, a linear model was used to assess the change in outcomes at post-acute exercise relative to baseline by performing paired t-tests. All *in vitro* experiments were performed in biological triplicate and pooled, allowing statistical analysis to be performed. Shapiro-Wilks test of normality were performed. All data was determined to be normally distributed. One-way ANOVAs were performed using GraphPad Prism Version 9.0.1 (GraphPad Software Inc, USA) to determine differences between DMSO vehicle control, chemotherapy without serum conditioning (results taken from *Chapter 4*) and both serum conditions (pre- and post-acute exercise) on endothelial apoptosis, eNOS, ICAM-1 expression and wound healing (AUC, $V_{migration}$, $T_{1/2\ gap}$) for each of the drug conditions (FEC and docetaxel). Statistical significance was assumed if $p < 0.05$.

6.3 Results

6.3.1 Participant Characteristics and Heart Rate Response to Exercise

Three healthy females were recruited based on inclusion-exclusion criteria. Participants were 26.0 ± 4.32 years, BMI: 22.3 ± 1.84kg·m^2, participated in 98 ± 20 minutes/week moderate-vigorous physical activity, and $\dot{V}O_{2peak}$ was 35 ± 2.16 ml·kg·min^{-1} **(Table 1)**.

Table 6.1. Participant characteristics and heart rate response during acute exercise

Characteristic	**Participants (n=3)**
Age (years)	26.0 ± 4.32
BMI (kg/m^2)	22.3 ± 1.84
MVPA (minutes/week)	98 ± 20
$\dot{V}O_{2peak}$ (ml/kg/min)	35.36 ± 2.16
Average Heart Rate (bpm)	117.8 ± 18.2
Average Heart Rate (%HR_{max})	57.9 ± 12.6

Data displayed are Mean ± SEM.

6.3.2 Pre- and Post-Acute Exercise Serum Preconditioning on Effects of FEC-T on HCAEC Morphology

From cell images, control HCAECs with both pre- and post-acute exercise serum preconditioning are live and growing with the expected morphology. With FEC-T drug exposure, there is a reduced number of adherent cells with a morphology which has changed to more shrunken cells which tend to clump together. This is similar for both pre- and post-acute exercise serum (**Figure 6.2**).

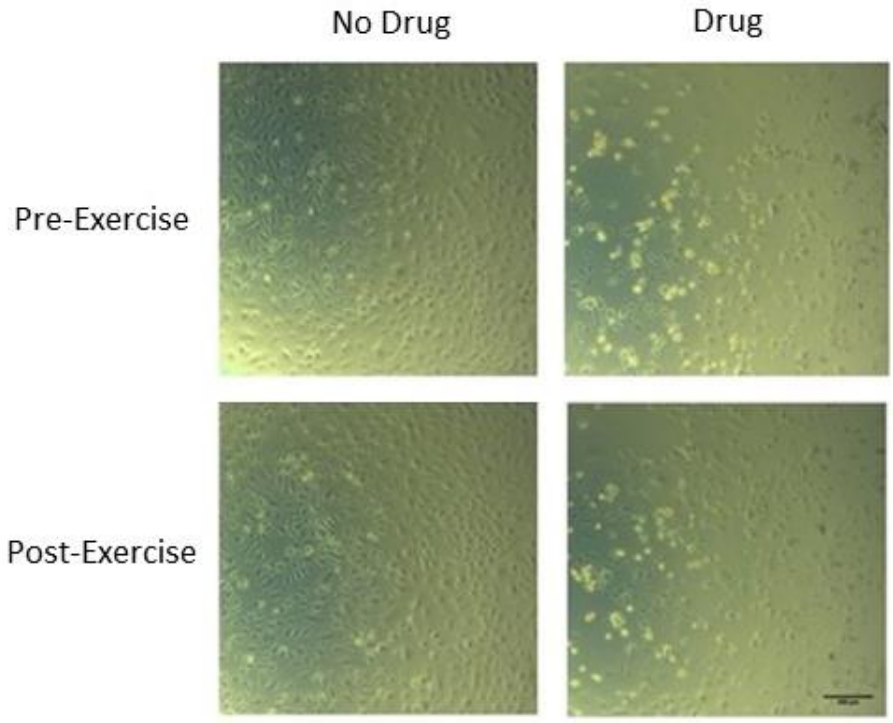

Figure 6.2. Representative images of HCAECs conditioned with pre- and post-acute exercise serum with and without chemotherapy drug exposure. *Images are X40 magnification. Scale bar is 250µm.*

6.3.3 Effects of Pre- and Post-Acute Exercise Serum Preconditioning on Annexin-V and Cleaved Caspase-3 Expression After Exposure to FEC-T Drugs

One-way ANOVAs revealed some statistically significant differences between exercise pre- and post-acute exercise serum preconditioning on the effects of FEC exposure on HCAECs (**Figure 6.3**). At 4-hours post exposure to FEC there was a significantly lower expression of Annexin V with post-exercise serum (39.2 ± 1.42%) compared to pre-exercise serum preconditioning (45.3 ± 1.47%, p = 0.020) (**Figure 6.3A)**. However, there was no significant between-group difference for cleaved caspase-3 expression (**Figure 6.3B**). Similarly, there was no significant between-group difference found for docetaxel exposure for Annexin V or cleaved caspase-3 expression (**Figure 6.3C and D**).

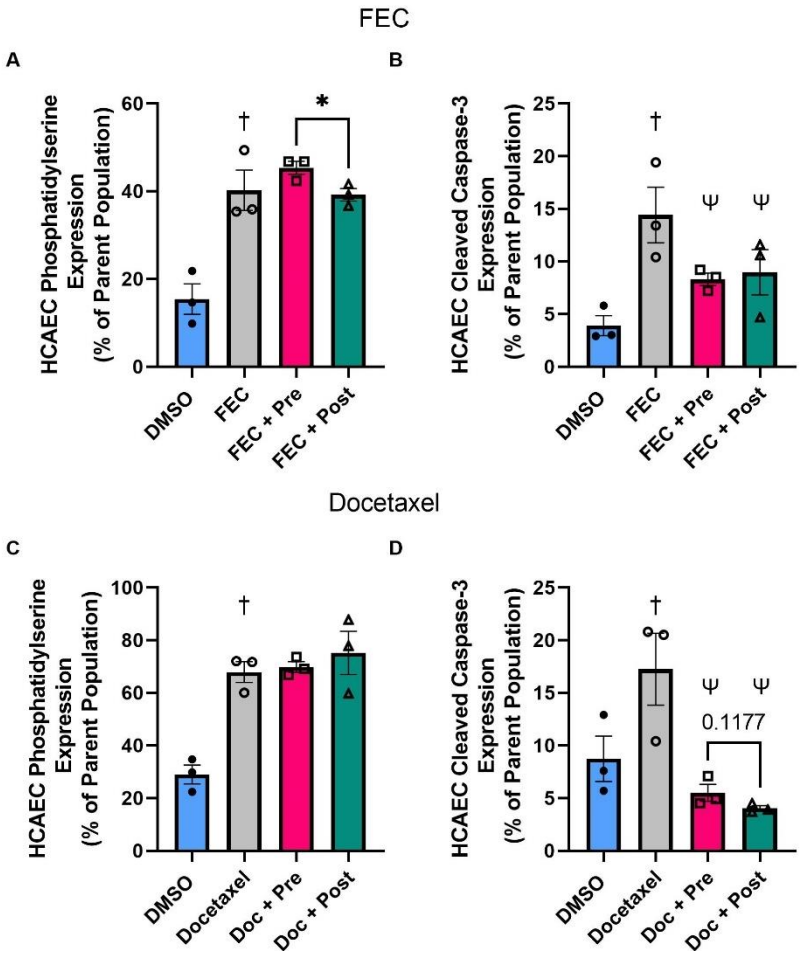

Figure 6.3. Comparison of pre- and post-acute exercise serum preconditioning on the effects of FEC (A&B: 5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) and 6µM docetaxel (C&D) exposure on HCAEC phosphatidylserine (A, C) and cleaved caspase-3 (B, D) expression at 4- and 48-hours post-exposure, repsectively, as determined by ANOVA. *Data shown are mean ± SEM (n=3). * p < 0.05. * p < 0.05. *p <0.05 pre- to post-group difference, † significant compared to DMSO, Ψ significant compared to drug only condition.*

6.3.4 Effects of Pre- and Post-Acute Exercise Serum Preconditioning on eNOS Expression After Exposure to FEC-T Drugs

HCAEC eNOS expression was the same for both groups after exposure to FEC combined and docetaxel drugs, with one-way ANOVAs showing no statistical differences between pre- vs post-exercise serum preconditioning (**Figure 6.4**).

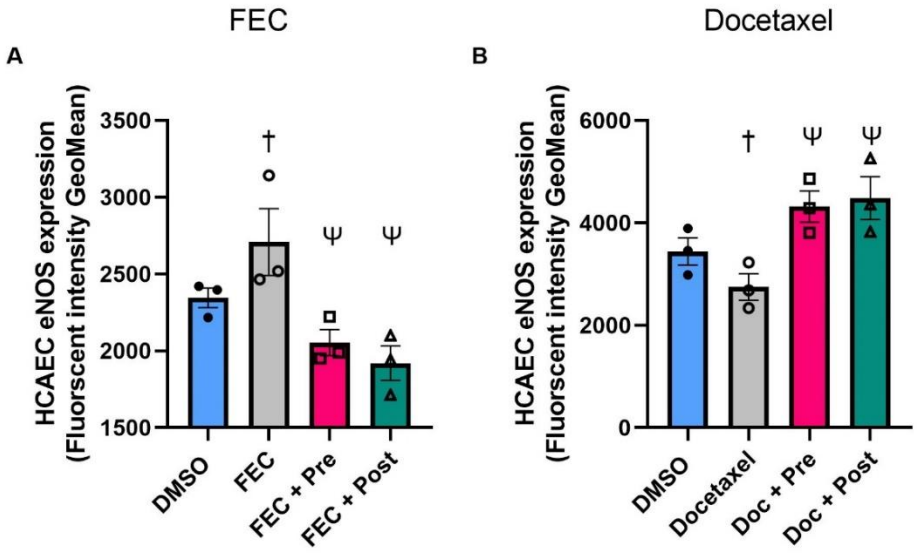

Figure 6.4. Comparison of pre- and post-acute exercise serum preconditioning on the effects of FEC (A: 5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) and 6µM docetaxel (B) exposure on HCAEC eNOS expression at 4- and 48-hours post-exposure, respectively, as determined by ANOVA. *Data shown are mean ± SEM (n=3). * p < 0.05. *p <0.05 pre- to post-group difference, † significant compared to DMSO, Ψ significant compared to drug only condition.*

6.3.5 Effects of Pre- and Post-Acute Exercise Serum Preconditioning on ICAM-1 Expression After Exposure to FEC-T Drugs

With FEC exposure, there was no between-group difference when HCAECs were exposed to FEC (sedentary: 1083 ± 86 MFI; active: 1207 ± 74 MFI) (**Figure 6.5A**). However, there was a significantly higher expression of ICAM-1 in HCAECs preconditioned with post-acute exercise serum (862 ± 37 MFI) when compared to those preconditioned with serum taken before an acute exercise bout (735 ± 28 MFI, p = 0.012) (**Figure 6.5A**).

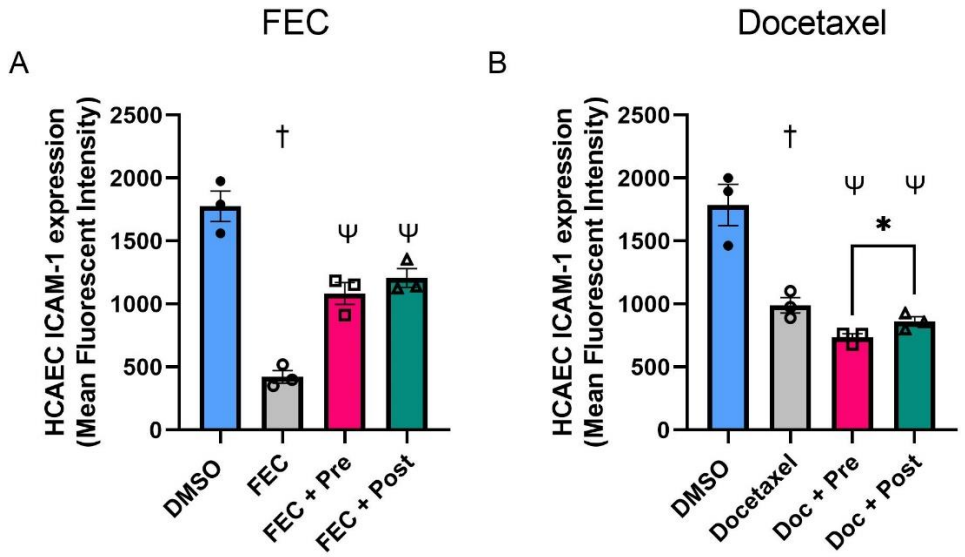

Figure 6.5. Comparison of pre- and post-acute exercise serum preconditioning on the effects of FEC (A: 5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) and 6µM docetaxel (B) exposure on HCAEC and ICAM-1 expression at 4- and 48-hours post-exposure, respectively, as determined by ANOVA. *Data shown are mean ± SEM (n=3). * p < 0.05. *p <0.05 pre- to post-group difference, † significant compared to DMSO, Ψ significant compared to drug only condition.*

6.3.6 Effects of Pre- and Post-Acute Exercise Serum Preconditioning on HCAEC Wound Repair

Representative images of HCAEC wound healing comparing pre- and post-acute exercise serum preconditioning are presented in **Figure 6.6**. Comparison of gap closure over time and area under the curve relative to wound closure over time are presented in **Figure 6.7** and **6.8**, respectively. Analysis of HCAEC wound healing assay revealed no between-group differences for $V_{migration}$, area under the curve or $T_{1/2gap}$ for any of the chemotherapy drug conditions. Full results are displayed in **Appendix 18**.

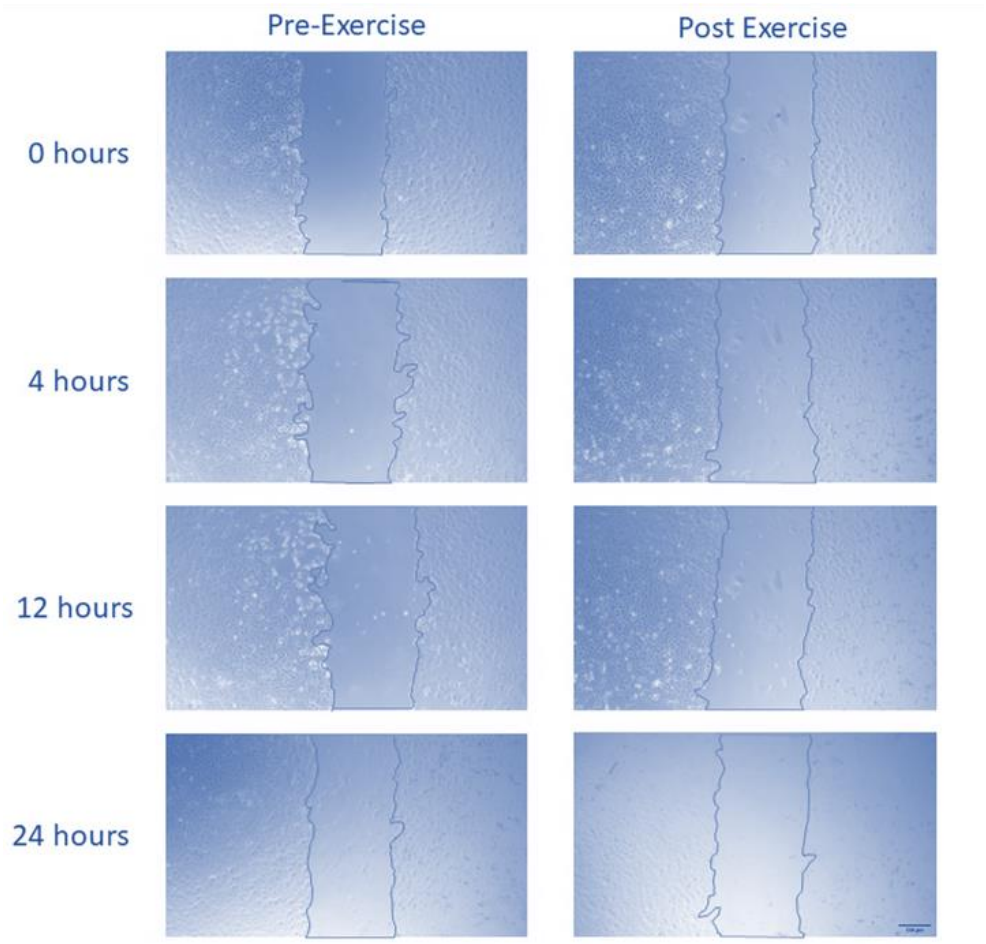

Figure 6.6. Representative images of HCAEC gap closure over time comparing pre- and post-acute exercise serum preconditioning. *Images are X4 magnification. Scale bar is 250µm.*

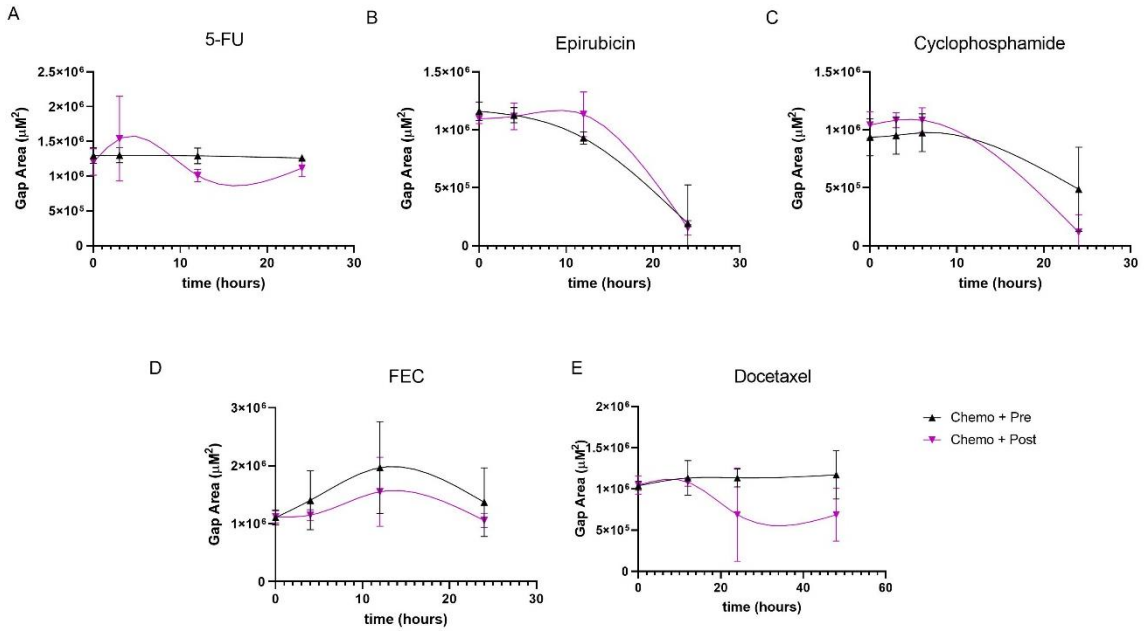

Figure 6.7. HCAEC wound closure (Gap area (μM^2)) over time (hours) with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), FEC combined (D), and docetaxel (E) comparing pre- and post-acute exercise serum preconditioning. *Data are presented as Mean ± SEM* (n=3).

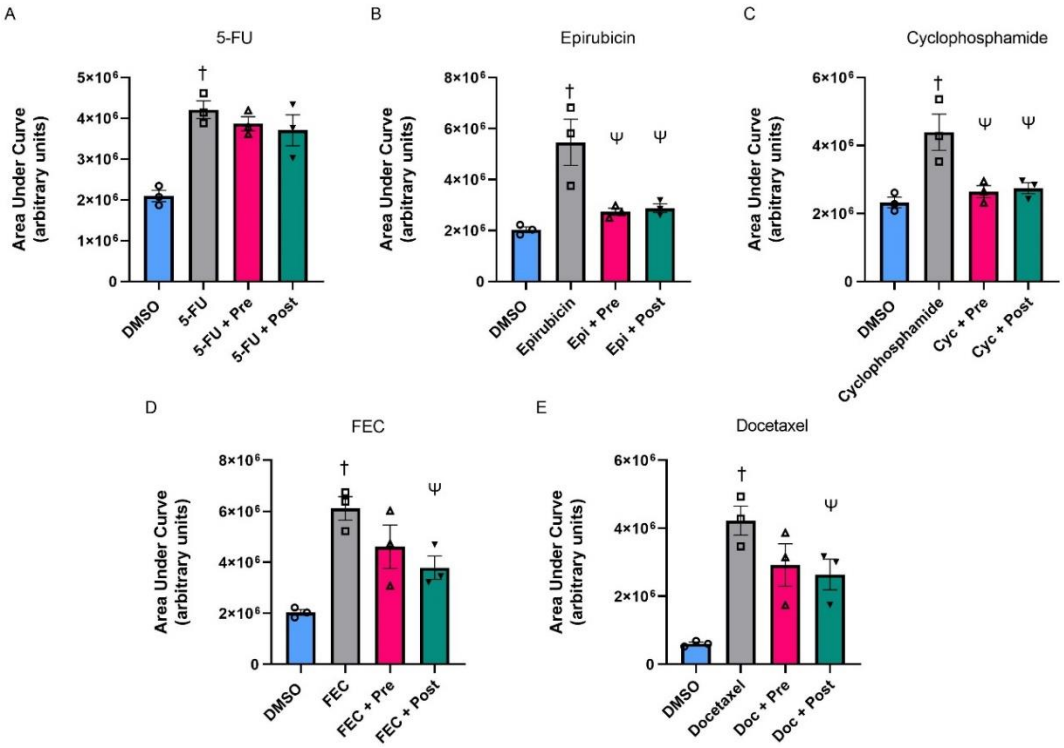

Figure 6.8. Area under the HCAEC gap closure curve with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), FEC combined (D), and docetaxel (E) treatments with DMSO, drug without serum, pre-exercise training serum with drug and post-exercise training serum, with drug, as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p <0.05 pre- to post-group difference, † significant compared to DMSO, Ψ significant compared to drug only condition.*

6.4 Discussion

Acute exercise offers significant vascular endothelial protection from FEC exposure, as shown by the 15% reduction in HCAEC phosphatidylserine expression (as indicated by Annexin V binding) with serum preconditioning post-exercise compared with pre-exercise serum **(Figure 6.3A)**. This is likely due to an increase in anti-inflammatory factors such as IL-6, IL-1ra, and IL-10 (Petersen et al., 2011). During exercise, contracting muscle cells upregulate gene expression and secretion of anti-inflammatory myokines, including IL-6, into the circulation, with subsequent stimulation of IL-1ra and IL-10 release from monocytes (Petersen et al., 2011), which block the pro-inflammatory effects of IL-1α and IL-1β and inhibit the production of these pro-inflammatory factors, respectively (Petersen et al., 2011). This is important for endothelial cells as inflammation is a stimulus for endothelial cell activation and apoptosis (Joussen et al., 2009), and therefore, may be a contributing factor to the reduction in early apoptosis observed with post-exercise serum conditioning **(Figure 3A)**. Furthermore, acute exercise enhances skeletal muscle glucose uptake as glucose is utilised as an energy substrate during exercise, resulting in reduced blood glucose concentrations from pre- to post-exercise (Lira et al., 2017). As high circulating glucose levels have detrimental effects on endothelial cells, acute exercise-induced reductions in glucose may also have contributed to the reduced endothelial cell apoptosis when exposed to FEC chemotherapy (Altannavch et al., 2004; Risso et al., 2001). However, there is no protection offered from acute exercise before docetaxel exposure (**Figure 6.3C&D)**. Nonetheless, the protection against FEC vascular toxicity may have clinically meaningful implications as if findings translate to in-human studies, the reduction in endothelial cell death may maintain integrity of the endothelial layer upon exposure to chemotherapy. This is essential to protect against cardiovascular toxicity of cancer treatment as the endothelium plays a key role in reducing CVD development (Verhamme et al., 2016). The 13% reduction in early apoptosis found in the present study is important as *in vivo*, endothelial damage as assessed by CECs is indicative of CVD status, with significantly higher CECs in individuals with vascular disease (Makin, Blann, Chung, Silverman, & Lip, 2004). Therefore, reducing endothelial injury and apoptosis with a single exercise bout would reduce the risk of future development of CVD which is prevalent in BC survivors (Kalábová et

al., 2011). Repeated single exercise bouts before each chemotherapy treatment is perhaps a therapeutic method to reduce endothelial toxicity (as shown in the current study) and has been utilised within cancer care with the aim to reduce cardiovascular toxicity (Kirkham et al., 2017). Therefore, the current findings provide mechanistic evidence to strengthen the rationale for acute exercise before chemotherapy exposure.

The current findings are novel and there is no other study which has utilised the same acute exercise serological preconditioning model in chemotherapy toxicity by which to compare results. Other *in vitro* studies using serum preconditioning for oncological research have investigated acute exercise effects on cancer cell growth and have not investigated cardiotoxicity, despite the pressing importance to tackle this clinical issue in cancer care. Acute exercise serum preconditioning reduces cancer cell growth by ~9%, likely due to changes in humoral and immune factors, such as epinephrine, TNF-α, IL-6, and IL-8 which inhibit proliferative signalling (Metcalfe et al., 2021; Orange et al., 2020). As ours is the first known study to utilise this same method in cardiotoxicity research, this adds to the literature that acute exercise not only can improve tumour outcomes but can also reduce vascular toxicity of chemotherapy.

With regards to in-human studies, there is now emerging evidence that participating in an acute exercise bout in the first week of the first chemotherapy cycle may reduce side effects, including fatigue and nausea, improving treatment tolerance (Johnsson et al., 2019). However, evidence of acute exercise prior to chemotherapy as a strategy to reduce cardiotoxicity of treatment is limited. There is currently only one study using cardiovascular outcome measures to investigate effects of an acute exercise bout 24-hours before chemotherapy for BC, finding that exercise decreased systemic vascular resistance and improved cardiac function, associating with a 46% risk reduction of heart failure, as indicated by circulating NT-proBNP at 14-days post-treatment (Kirkham et al., 2017). Although this is the only in-human study of its kind, these findings are promising. However, this was a small trial lacking measures of vascular function and therefore, there is a requirement for larger scale trials investigating acute exercise effects on vascular toxicity. Our findings add to the literature that acute exercise protection also occurs in vascular endothelial cells and this is potentially a mechanism behind the reduction in HF risk, as endothelial detriments are often initiating steps in CVD development (Verma et al., 2003).

The majority of evidence for 'prehabilitation' exercise comes from rodent studies. Taken together, the evidence for exercise 'prehabilitation' in animal models is promising, with maintenance of cardiac function, likely due to preserved contractile properties, reduced oxidative stress, protection of mitochondrial health, reduced apoptosis, and improved endothelial function (Chen, Wu, Middlekauff, & Nguyen, 2017). For example, BC rats which performed a single aerobic exercise bout (60-minutes, 25m/min) 24 hours before doxorubicin treatment had significantly higher end-systolic pressure and LV developed pressure, compared with sedentary BC rats receiving doxorubicin without exercise preconditioning, indicating exercise-induced cardiovascular protection (Wonders et al., 2008). However, no animal study of acute exercise before chemotherapy exposure has investigated vascular outcomes measures. Now, we have provided *ex vivo* evidence from human participants that an acute exercise bout can reduce FEC-induced cell death by 15% (**Figure 6.3A**), further strengthening the findings of cardiovascular protection offered by acute exercise in rodent studies.

Since evidence from cancer populations is limited, it is important to draw comparisons from non-cancer studies of acute exercise and the endothelium. *In vitro* studies found that mimicking exercise with laminar shear stress on cultured endothelial cells suppresses apoptosis (Dimmeler, Hermann, Galle, & Zeiher, 1999) and reduces endothelial microparticles (EMPs) release – markers of endothelial damage (J. S. Kim et al., 2015). The current serological study observed reductions in endothelial apoptosis after acute exercise **(Figure 6.3A)**, adding to the literature that changes to the serological environment with acute exercise are also protective to the endothelium upon exposure to chemotherapy. When combined with shear stress, this may well produce an additive effect, enhancing the protecting effects of exercise on the vasculature. In this study, effects on endothelial cells are due to acute exercise-induced alterations in circulating factors alone. However, the specific factors involved are yet to be investigated. Findings that acute exercise reduces endothelial apoptosis contrasts in-human findings that there are no changes in CECs or circulating EMPs immediately following acute exercise (Sapp et al., 2019; Sapp & Hagberg, 2018). However, these studies were not investigating endothelial injury in response to chemotherapy exposure and therefore, acute exercise may only decrease apoptosis when the apoptotic stimulus (chemotherapy) is present. Reduction in endothelial cell apoptosis

found in our study may be explained by serological inhibition of the apoptotic response to toxic environmental stimuli. In rodent studies, acute exercise activates NF-κB signalling pathway in skeletal muscle, accompanied by a cascade of events, including increased nuclear P50 content (Ji, Gomezcabrera, Steinheafel, & Vina, 2004). This P50 expression is anti-apoptotic, inducing cell survival (Yu, Wan, & Huang, 2009). Therefore, anti-apoptotic effects of acute exercise may be due to a serological stimulus which activates NF-κB, resulting in activation of the *p*50 pathway. Current findings of a 15% reduction in endothelial cell apoptosis after FEC exposure adds to the literature that serological conditioning of endothelial cells post-acute exercise, in absence of shear stress, can protect against FEC-induced apoptosis, highlighting the importance of systemic alterations with acute exercise. However, it is important to note that this may be a relatively small effect and its clinical relevance remains to be investigated. Additionally, although there was no protective effect of acute exercise on docetaxel-induced apoptosis, current findings are still promising.

The endothelium plays a key role in reducing CVD development by offering anti-thrombotic properties, including NO production, levels of which are inferred by FMD (Verhamme et al., 2016). Results from our study show that eNOS (the key enzyme responsible for NO production) expression did not change with acute exercise in either FEC or docetaxel exposed HCAECs **(Figure 6.4)**. As there is a paucity of evidence in cancer populations, is important to look at non-cancer studies of acute exercise and endothelial function to determine context for the current results. With acute exercise, there is an immediate decrease in FMD, followed by a (supra-) normalization response, likely mediated by shear stress (Dawson, Green, Cable, & Thijssen, 2013). Acute-exercise changes in FMD differ across study populations and exercise protocols but the overall evidence is that acute exercise results in enhanced endothelial-dependent increases in NO bioavailability in at-risk populations (Farsidfar et al., 2008; Harris, Padilla, Hanlon, Rink, & Wallace, 2008; Zhu et al., 2010). This is important in those treated with chemotherapy, as enhanced NO bioavailability reduces atherosclerosis risk by preventing adhesion to the endothelium (Mudau et al., 2012a; Verhamme et al., 2016). However, our findings suggest that NO is not upregulated **(Figure 6.4)**, implying that exercise-induced alterations in the serum environment alone are not sufficient to induce alterations in endothelial NO production. This will not necessarily be

the case *in vivo* as exercise produces a shear stress stimulus which is responsible for upregulation of NO production (Boo et al., 2002).

This study found that acute exercise-induced alterations in the serum environment attenuated the reduction in endothelial cell surface ICAM-1 expression when exposed to docetaxel **(Figure 6.5B)** and were unchanged with exposure to FEC **(Figure 6.5A)**, despite the clear protective effects of exercise on endothelial apoptosis **(Figure 6.3A)**. The acute-exercise-induced increase in expression of ICAM-1 corresponds to the literature that acute exercise in humans upregulates ICAM-1 (Tinken et al., 2010). This is thought to be due to the shear stress stimulus as cultured endothelial cells exposed to shear stress also increase ICAM-1 expression (Chiu et al., 2004). Therefore, our findings now add to the literature that serological alterations are also likely involved in ICAM-1 upregulation with acute exercise. Chemotherapy is known to upregulate adhesion molecules (Antonella De Angelis et al., 2017; Baeten et al., 2006). However, our findings from *Chapter 4* suggest that this is not the case, with differences likely due to timing of analysis (Meijer, 2009) and/or detection methods (Volanti et al., 2004). Regardless, the results from this acute exercise study suggest that acutely, exercise attenuates the reduction in ICAM-1 with docetaxel exposure. A non-chemotherapy *ex vivo* study investigating systemic effects of a single exercise session on adhesion molecules (also in the absence of shear stress) found that acute exercise reduced peripheral blood mononuclear cell-HUVEC adhesion by ~81%, independent of ICAM-1 expression, suggesting that immune cells have a reduced ability to adhere to the endothelium after exercise (Mills et al., 2006). Therefore, it may be changes to immune cell function and not to endothelial cell adhesion molecule expression that reduces risk of atherosclerosis development with an exercise bout. No study has investigated acute exercise effects on endothelial adhesion molecule response to chemotherapy and hence, results that ICAM-1 upregulation is enhanced with exercise preconditioning before FEC exposure and unchanged with exercise preconditioning before docetaxel **(Figure 6.5)** are novel.

Lastly, there was no significant effect of acute exercise serum preconditioning for any of the FEC-T drug conditions on endothelial repair. A non-chemotherapy *ex vivo* study also found no between-group difference for pre- and post-acute exercise on endothelial wound repair (Wahl et al., 2014). This is despite enhanced concentrations of VEGF in the post-exercise

serum, with VEGF identified as key for inducing HCAEC migration (Wahl et al., 2014). Therefore, there must be other factors which inhibit the VEGF-induced endothelial migratory response with acute exercise serum preconditioning. On the other hand, it may well be the case that the current acute exercise session was not of a sufficient intensity to produce an effective pro-angiogenic serological response. VEGF release from muscle cells into the circulatory system during an exercise bout is intensity dependent (Wahl et al., 2011). Similarly, endothelial microvesicles have also been identified as important for acute exercise-induced upregulation of angiogenesis and, likewise, their release is exercise intensity-dependent (Wilhelm et al., 2016). In this study, the average HR was 57% of HR_{max} (**Table 6.1**) and perhaps a higher intensity exercise session is required for effects to be observed. The next step for this research is to determine whether a longer exercise intervention can offset the detrimental effects of chemotherapy on endothelial wound repair. Obtaining this information is crucial for the field of exercise cardio-oncology as endothelial damage without efficient repair is an initiating step in atherosclerosis development (Sullivan et al., 2000; Verma et al., 2003) and may be a contributing factor to cardiovascular toxicity observed in BC populations (Patnaik et al., 2011a; Yeh & Bickford, 2009).

6.5 Conclusions and Implications

To conclude, the current study has investigated a variety of potential targets for acute exercise protection against endothelial toxicity of FEC-T chemotherapy. The current findings provide the first *in vitro* evidence that inactive individuals can alleviate their risk for vascular toxicity by performing a single exercise session before the first chemotherapy cycle. This is observed by a reduction in HCAEC Annexin V binding to phosphatidylserine in response to FEC drugs with preconditioning endothelial cells with serum after a single exercise session. However, this study found no difference in apoptotic state of HCAECs between pre- and post-exercise conditioning with docetaxel. Likewise, there was no between-group difference for changes in eNOS expression for either FEC or docetaxel conditions, suggesting that protective effects of serological preconditioning are independent of NO pathways; and that *in vivo* upregulation of eNOS with acute exercise perhaps requires a shear stress stimulus (Boo et al., 2002). Additionally, the upregulation of ICAM-1 with acute exercise in response to FEC exposure poses as an interesting phenomenon which cannot yet be fully explained.

Potential mechanisms of acute exercise on the endothelium have previously been thought to be shear stress-mediated (Boo et al., 2002) but now, for the first time, we have shown that systemic circulatory changes can help to prevent endothelial cell death s in response to chemotherapy.

Chapter 7

Investigation of the potential for protective effects an exercise training intervention on the toxicity of FECT chemotherapy on HCAECs

7.1 Introduction

Study *Chapters 4, 5 and 6* demonstrate that (1) chemotherapy induces toxic effects of human endothelial cells, and that both physical activity status (2) and (3) acute exercise can modulate the chemotherapy-induced apoptosis, eNOS and ICAM-1 downregulation, and wound repair inhibition on these vascular cells. As these findings show that there is promise that physical activity and/or exercise may alleviate chemotherapy-induced vascular toxicity, there is a requirement to determine whether an exercise intervention can elicit protection against FEC-T chemotherapy toxicity. The main question arising from findings in *Chapter 5* is whether physically active female serum protection of endothelial cells is due to the regular exercise or other dietary/lifestyle habits? The effects of regular exercise will now be investigated by undertaking an exercise trial in previously sedentary females.

There is evidence suggesting the use of exercise interventions during chemotherapy treatment against CV toxicity in BC survivors, with exercise interventions found to reduce CVD risk by 20%, determined by improvements in vascular health (Lewington et al., 2002; Sturgeon, Ky, Libonati, & Schmitz, 2014a). Specifically, there is an 11% decrease in risk of developing CVD in BC survivors participating in a 16-week exercise intervention after completion of chemotherapy treatment, as determined by Framingham Risk Scores (Lee et al., 2019). These studies did not include direct measurements of vascular endothelial function and hence, conclusions regarding effects on the vascular endothelium cannot be directly drawn. However, two known studies have investigated effects of exercise during chemotherapy on measurements of vascular endothelial function (FMD) but have provided contrasting findings (Jones et al., 2013; Lee et al., 2019b). Therefore, evidence is insufficient to provide a conclusion regarding an exercise intervention on chemotherapy effects on vascular endothelial health and its involvement in protecting against CVD risk. No study has investigated vascular protective effects of an exercise intervention prior to chemotherapy treatment. There is still a requirement to determine whether an intervention before treatment can elicit vascular protection against chemotherapy and perhaps further boost the protection of an exercise intervention throughout the duration of chemotherapy cycles and into survivorship by preconditioning the vasculature to be protected against initial chemotherapy damage.

As there are some promising findings from *Chapter 5* and *Chapter 6*, a longitudinal study design has now been chosen to progress the research to determine whether people who are 'at-risk' of CVD can be conditioned by an exercise intervention to become less of a risk for chemotherapy-associated CVD. The aim of this study is therefore to determine whether an exercise intervention prior to FEC-T chemotherapy elicits vascular protection at the endothelial cell level by assessing endothelial cell apoptosis, eNOS and adhesion molecule expression in a HCAEC model. It is hypothesised that the exercise training intervention will result in protection against the usual FEC-T induced endothelial damage and dysfunction.

7.2 Methods and Materials

All participants provided written informed consent, and the study was approved by the Liverpool Central NHS Research Ethics Committee (approval reference no. 17/NW/0042) and conformed to the Declaration of Helsinki. This is a collaborative study between Edinburgh Napier University and Liverpool John Moore's University. Therefore, the study was also approved by Edinburgh Napier University Research Integrity Committee and Liverpool John Moore's University.

7.2.1 Participants

The study followed the process in place for the Active Lifestyles Exercise Referral Scheme (ERS) within the Metropolitan Borough of Sefton, in the Northwest of England, funded by Sefton Clinical Commissioning Group (CCG). Patients were either referred to the Active Lifestyles ERS by their GP or self-referred following advertisement in their local area. Most were sent an email via their occupational health officer with a poster attached. Following initial statement of interest, participants were recruited from the Active Lifestyles Exercise Referral Scheme via their GP. Participants who had hypertension, angina (treated and stable), anxiety, stress, depression, arthritis, previous heart attack/surgery (not under current investigation), impaired glucose tolerance, overweight/obese (BMI >25kg·m^2), aged 18-65 years, dyslipidaemia (total cholesterol >5mmol·L^{-1}) were included in this study. However, people excluded were those undergoing cardiac rehabilitation, blood pressure >180/100mmHg and/or uncontrolled/poorly controlled hypertension, currently taking β-blockers, or presenting with cardiomyopathy, uncontrolled tachycardia, arrhythmia, valvular

heart disease, aneurysms, uncontrolled epilepsy, history of falls/dizzy spells in last 12 months, excessive/unexplained breathlessness, uncontrolled/poorly controlled asthma/severe COPD, pregnant or breast feeding, end stage renal disease, awaiting medical investigation, severe mental health condition, diabetes plus one of the following: aged >35, T2 diabetes mellitus 10 years, T1 diabetes mellitus>15 years, hypercholesterolemia (total cholesterol > 6.2mmol·L^{-1}), hypertension, smoking, familial history of CAD, microvascular disease, peripheral vascular disease, autonomic neuropathy.
A total of 160 potential participants were contacted, 73 were eligible for participation and consented to the study. 38 withdrew from the study for the following reasons: injury (n=5), ill-health (n=3), work commitment (n=8), family issues (n=6), moved (n=1), lost contact (n=15); and 15 withdrew at follow-up for the following reasons: injury (n=1), ill-health (n=1), work commitment (n=7), family issues (n=2), lost contact (n=4).

(e.g., number contacted, number recruited, number lost to follow up), and exercise (e.g., how exercise adherence was monitored.

7.2.2 Baseline Measurements

All participants attended the laboratory at LJMU following an overnight fast, having abstained from caffeine, alcohol, and exercise the day prior. Following 15 minutes of supine rest, brachial artery blood pressure was measured in triplicate using a sphygmomanometer (Dianamap; GE Pro 300V2, Tampa, Florida). Body composition was analysed using Dual-energy X-ray Absorptiometry (DXA Hologic QDR Series, Discovery A, Bedford, MA, USA). Resting blood samples were obtained, as detailed in *Chapter 5*.

7.2.3 Protocol for Maximal Testing

After blood sample collection, an incremental exercise test to exhaustion was performed on an electromagnetically braked cycle ergometer. Briefly, patients began cycling at 25W for 3 minutes, and workload increased by 35W every 3 minutes until volitional exhaustion. $\dot{V}O_{2peak}$ was determined using an online gas collection system (Moxus modular oxygen uptake system, AEI technologies, Pittsburgh, PA). $\dot{V}O_{2peak}$ was defined as the highest $\dot{V}O_2$ achieved over a 15 second recording period.

7.2.4 Exercise Training Intervention

To allow for sufficient recovery from maximal testing, the exercise training intervention commenced at least 72-hours after baseline assessments. Participants were encouraged to wear the HR monitor (Polar H10, Polar Electro Oy, Kempele, Finland) provided, throughout all sessions.

The intervention duration was 12-weeks and participants were encouraged to exercise 3 times per week. The sessions prescribed repeated 1-minute bouts of exercise interspersed with 1-minute of rest. Patients were advised to achieve ≥80% of predicted maximum HR (220-age) during the exercise intervals. The exercise intervals were composed of two different 30-second bodyweight exercises with no rest in between. During the first 2 weeks, patients were advised to complete 4 intervals. This increased by 1 interval every 2 weeks up to a maximum of 9 intervals. Patients were free to choose which bodyweight exercises they performed, according to the exercise pack provided which contained 9 exercise pairs (18 total individual exercises). Exercise ranged from low impact to complex movements with higher impact, allowing participants to modify sessions, choosing exercises which elicited the desired HR response, but were still suitable for level of mobility and fitness. This exercise protocol is outlined in **Figure 7.1**.

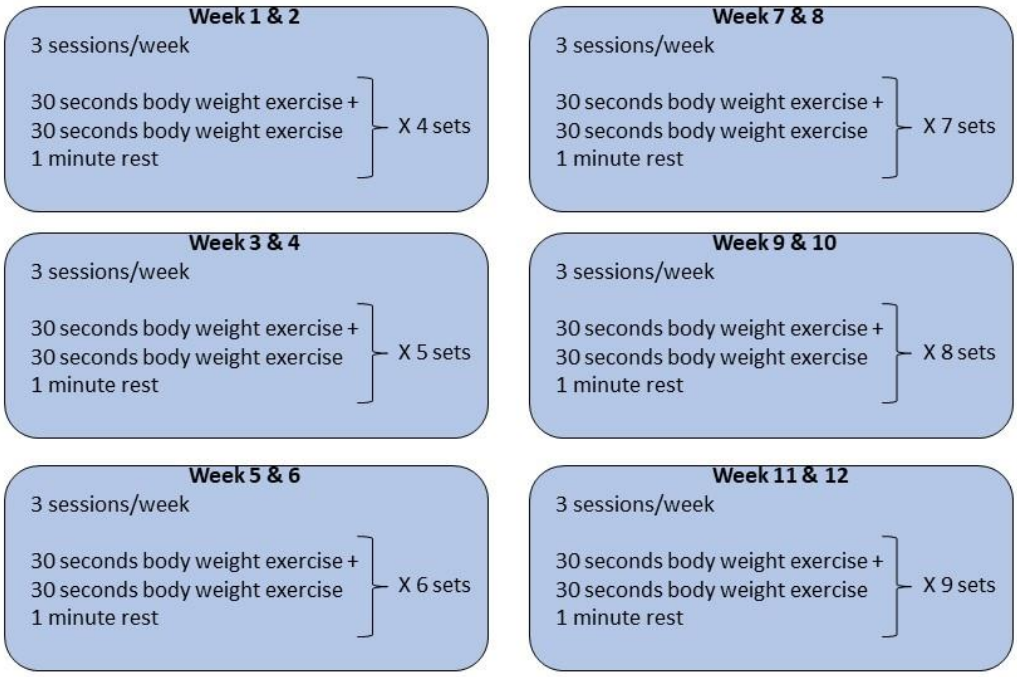

Figure 7.1. Prescribed home-based exercise intervention

As there are currently no specific exercise guidelines for those with BC to reduce vascular toxicity (Campbell et al., 2019b), the exercise protocol was chosen based on the HIIT intervention which improved FMD pre- to post-chemotherapy in BC patients (Lee, Kang, et al., 2019a). Furthermore, the rationale for choosing this specific HIIT training program is that it is similar to the acute exercise session (*Chapter 6*) in that it is a home-based exercise intervention. Therefore, giving comparability to the acute (*Chapter 6*) and chronic (*Chapter 7*) nature of these interventions.

Adherence to all sessions was monitored using the HR monitor (Polar H10, Polar Electro Oy, Kempele, Finland) which provided real-time feedback during sessions using the accompanying Polar Beat App (Polar Beat | Free fitness, running and workout app | Polar UK). Data was automatically uploaded to a cloud storage site accessible to the participant and research team throughout the intervention. Adherence and compliance were determined by the average HR during each session, number of intervals completed, peak HR on each interval, and % of intervals ≥80% HR_{max}.

7.2.5 Post-Intervention Measurements

Post-intervention measurements were conducted with identical procedures, methods, and timings to the baseline measurements. The post-intervention was collected at least 72 hours after the final training session to reduce any effects of acute exercise training on measurements.

7.2.6 Endothelial Cell Serum Preconditioning, Chemotherapy Exposure, and Analysis

The same HCAEC *ex vivo* serological conditioning model, flow cytometry and wound healing assay analysis was performed as in *Chapter 5* and *Chapter 6*.

7.2.7 Statistical Analysis

To assess exercise-induced changes in participant characteristics, a linear model was used to assess the change in outcomes at post-intervention relative to baseline within the treatment group by performing paired t-tests. All analyses were performed using GraphPad Prism Version 9.0.1 (USA). Statistical significance was assumed if $p<0.05$.

For *in vitro* analyses, all experiments were performed in biological triplicate and pooled, allowing statistical analysis to be performed. Shapiro-Wilks test of normality were performed. All data was determined to be normally distributed. One-way ANOVAs were performed using GraphPad Prism Version 9.0.1 (GraphPad Software Inc, USA) to determine differences between DMSO vehicle control, chemotherapy without serum conditioning (results taken from *Chapter 4*) and both serum conditions (pre- and post-exercise intervention) on endothelial apoptosis, eNOS, ICAM-1 expression and wound healing (AUC, $V_{migration}$, $T_{1/2\ gap}$) for each of the drug conditions (FEC and docetaxel). Statistical significance was assumed if $p < 0.05$.

7.3 Results

7.3.1 Participant Characteristics

Only participants who completed >75% of prescribed sessions were used for serological conditioning experiments, with an average adherence rate of 89 ± 9% of sessions completed. 5 participants were excluded due to poor adherence (average adherence rate of 45 ± 18% of sessions completed). There was no real difference in any of the physiological outcome measures between high and low adherence groups **(Table 7.1)**.

Participants were aged 53.18 ± 4.56 years. The exercise intervention had no significant effect on BMI, body fat, systolic BP, or diastolic BP (**Table 7.1**). However, the increase in pre- to post-$\dot{V}O_{2max}$ values were close to reaching significance in the high-adherence group (pre: 20.98 ± 4.99 ml·kg·min^{-1} vs post: 23.02 ± 5.79 ml·kg·min^{-1}, p = 0.053). Participants had an average of 1.7±0.8 underlying cardiovascular risk factors including hyperglycaemia, hypertension, obesity, hyperlipidaemia. Other medical conditions included hypothyroidism, acute urticaria, rosacea, arthritis, chronic kidney disease, auto immune hepatitis, and osteoarthritis.

Table 7.1. Participant characteristics before and after 12-week exercise intervention (n=15)

Characteristic	High Adherence Pre	High Adherence Post	*p*-value	Low Adherence Pre	Low Adherence Post	*p*-value
BMI (kg·m^2)	30.78 ± 4.81	30.72 ± 4.74	0.774	27.20 ± 3.06	26.86 ± 3.51	0.344
$\dot{V}O_{2max}$ (ml·kg·min^{-1})	20.98 ± 4.99	23.02 ± 5.79	0.053	21.24 ± 1.89	21.13 ± 2.83	0.649
Body fat (%)	37.99 ± 5.41	37.79 ± 4.96	0.708	38.82 ± 2.78	39.02 ± 2.77	0.865
Systolic BP (mmHg)	131 ± 15	126 ± 10	0.194	115 ± 8	115 ± 14	0.948
Diastolic BP (mmHg)	73 ± 8	73 ± 9	0.832	67 ± 6	72 ± 9	0.303
Mean Arterial Pressure (mmHg)	92 ± 10	90 ± 9	0.259	84 ± 6	87 ± 11	0.385

Values shown are mean ± SD

7.3.2 Pre- and Post-Exercise Training Serum Preconditioning on Effects of FEC-T on HCAEC Morphology

From cell images, control HCAECs with both pre- and post-exercise intervention serum preconditioning are alive and growing with the expected morphology. With FEC-T drug exposure, there is a reduced number of adherent cells with a morphology which has changed to more shrunken cells which tend to clump together but this effect is lessened in the post-exercise intervention serum preconditioned HCAECs compared to pre-exercise intervention (**Figure 7.2**).

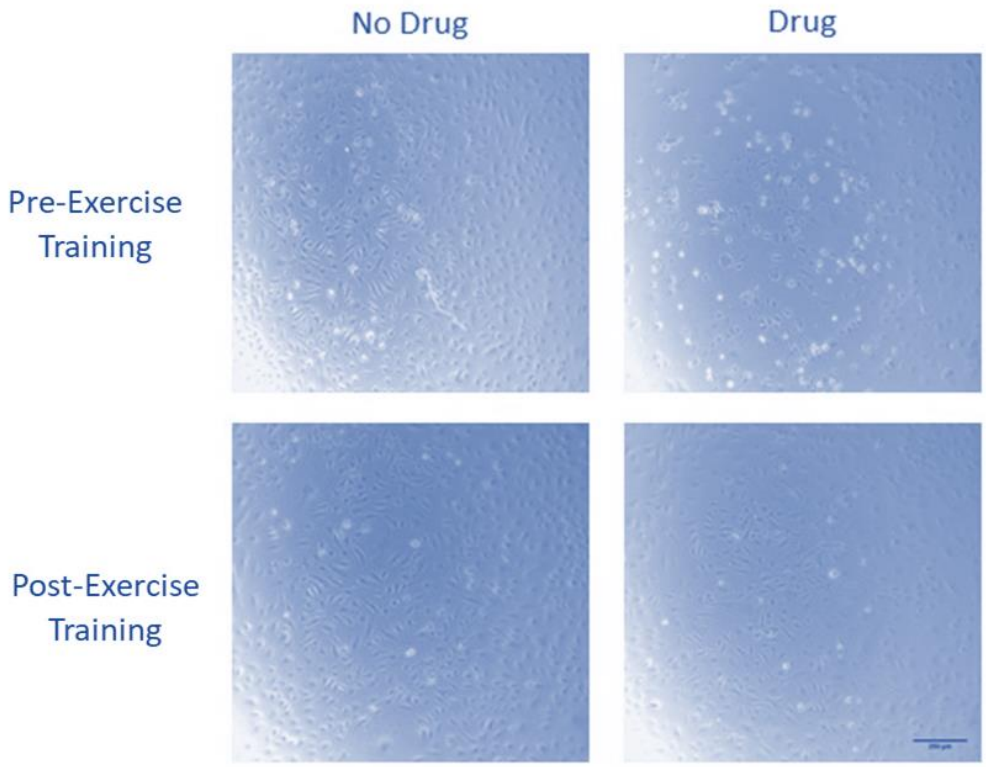

Figure 7.2. Representative images of HCAECs conditioned with pre- and post-exercise intervention serum with and without chemotherapy drug exposure. *Images are X4 magnification. Scale bar is 250µm.*

7.3.3 Effects of Pre- and Post-Acute Exercise Serum Preconditioning on Annexin-V, Cleaved Caspase-3, eNOS and ICAM-1 Expression After Exposure to FEC-T Drugs

One-way ANOVAs revealed significant differences for FEC combined treatments. Both Annexin V **(Figure 7.3A)** and cleaved caspase-3 **(Figure 7.3B)** expression decreased in the post-exercise serum preconditioning HCAECs (Annexin V pre: 42.23 ± 6.96% vs Annexin V post: 29.57 ± 7.09%, $p = 0.010$; cleaved caspase-3 pre: 6.13 ± 0.87% vs cleaved caspase-3 post: 4.43 ± 0.30%, $p = 0.022$). For docetaxel, there were no significant within-group differences for Annexin V or cleaved caspase-3 expression **(Figure 7.3C&D**). Full results are displayed in **Appendix 19.**

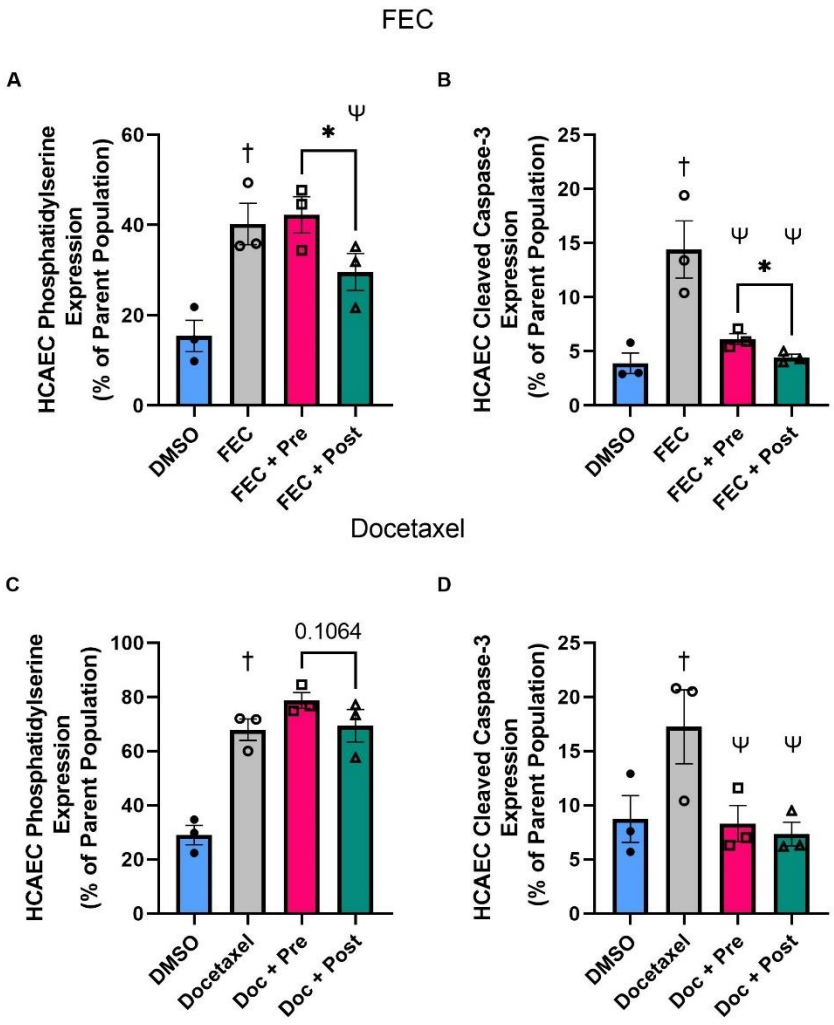

Figure 7.3. Comparison of pre- and post-exercise intervention serum preconditioning on the effects of FEC (A&B: 5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) and 6µM docetaxel (C&D) exposure on HCAEC phosphatidylserine (A, C) and cleaved caspase-3 (B, D) expressionat 4- and 48-hours post-exposure, repsectively, as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p <0.05 pre- to post-group difference, † significant compared to DMSO, Ψ significant compared to drug only condition.*

7.3.4 Effects of Pre- and Post-Exercise Intervention Serum Preconditioning on eNOS Expression After Exposure to FEC-T Drugs

There were no significant differences between pre- and post-exercise training serum for HCAEC eNOS **(Figure 7.4)** expression for FEC or docetaxel. Full results are displayed in **Appendix 19.**

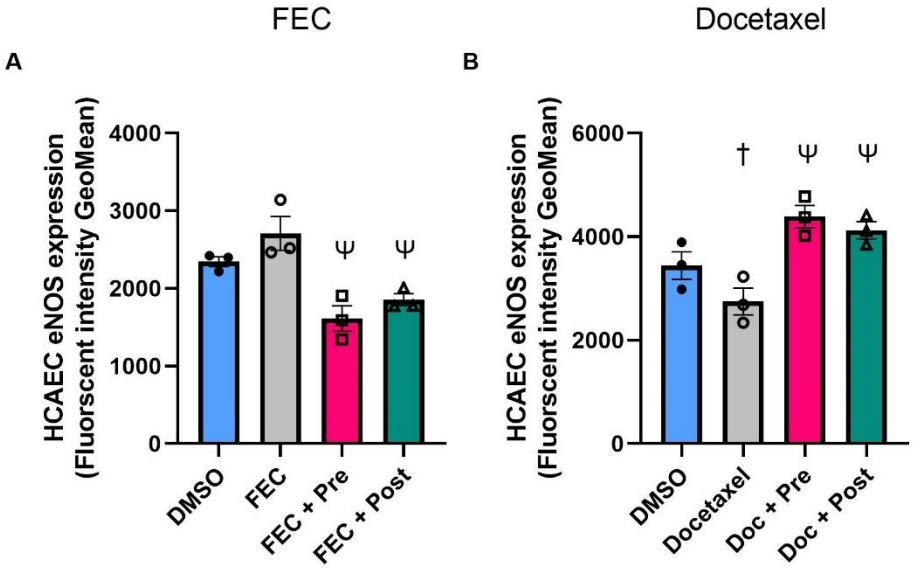

Figure 7.4. Comparison of pre- and post-exercise intervention serum preconditioning on the effects of FEC (A: 5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) and 6µM docetaxel (B) exposure on HCAEC eNOS expression at 4- and 48-hours post-exposure, respectively, as determined by ANOVA. *Data shown are mean ± SEM (n=3). † significant compared to DMSO, Ψ significant compared to drug only condition.*

7.3.5 Effects of Pre- and Post-Exercise Intervention Serum Preconditioning on ICAM-1 Expression After Exposure to FEC-T Drugs

There were no significant pre- to post-training differences for HCAEC ICAM-1 **(Figure 7.5)** expression for FEC or docetaxel.

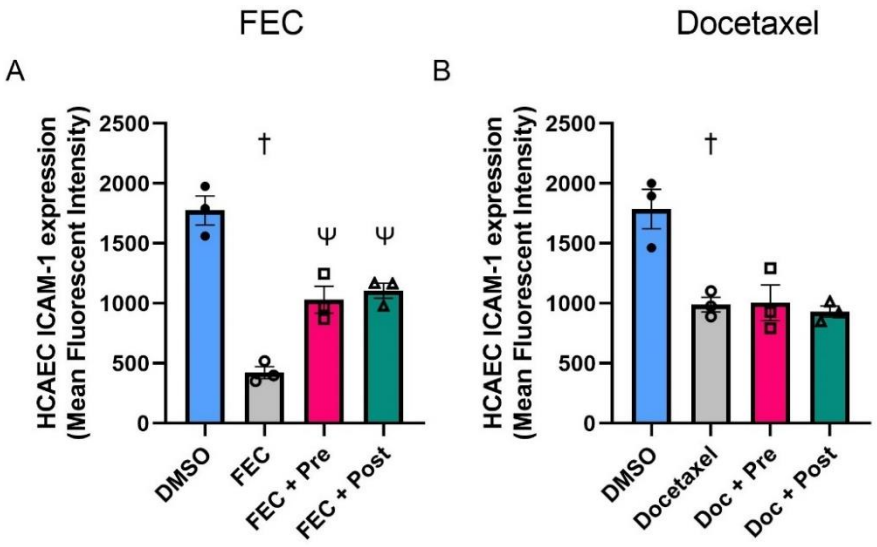

Figure 7.5. Comparison of pre- and post-exercise intervention serum preconditioning on the effects of FEC (A: 5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM) and 6µM docetaxel (B) exposure on HCAEC and ICAM-1 expression at 4- and 48-hours post-exposure, respectively, as determined by ANOVA. *Data shown are mean ± SEM (n=3). † significant compared to DMSO, Ψ significant compared to drug only condition.*

7.3.6 Effects of Pre- and Post-Exercise Intervention Serum Preconditioning on HCAEC Wound Healing

Representative images of HCAEC wound healing comparing pre- and post-exercise intervention serum preconditioning are presented in **Figure 7.6**. Comparison of gap closure over time and area under the curve relative to wound closure over time are presented in **Figure 7.7** and **7.8**, respectively. Analysis of HCAEC wound healing assay revealed significantly smaller AUC in favour of the post-exercise intervention group when exposed to 5-FU (p = 0.03) and a significantly faster rate of wound closure in favour of the post-exercise intervention group when exposed to docetaxel ($V_{migration}$ = 5.21 ± 1.82 µm/hr vs 0.36 ± 0.33 µm/hr, p = 0.02). There were no other between-group differences for $V_{migration}$, area under the curve or $T_{1/2gap}$ for any of the other chemotherapy drug conditions. Full results are displayed in **Appendix 20**.

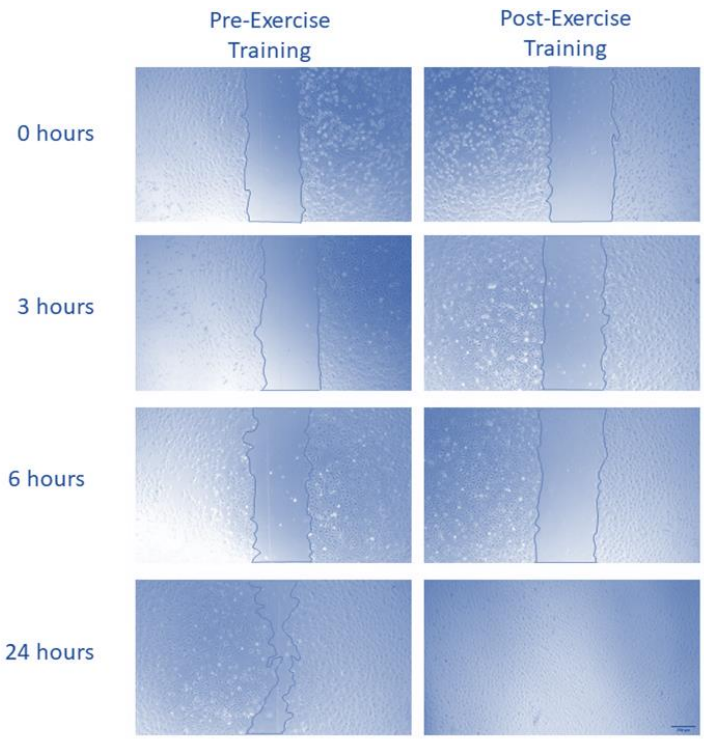

Figure 7.6. Representative images of HCAEC gap closure over time comparing pre- and post-exercise training intervention serum preconditioning. *Images are X4 magnification. Scale bar is 250µm.*

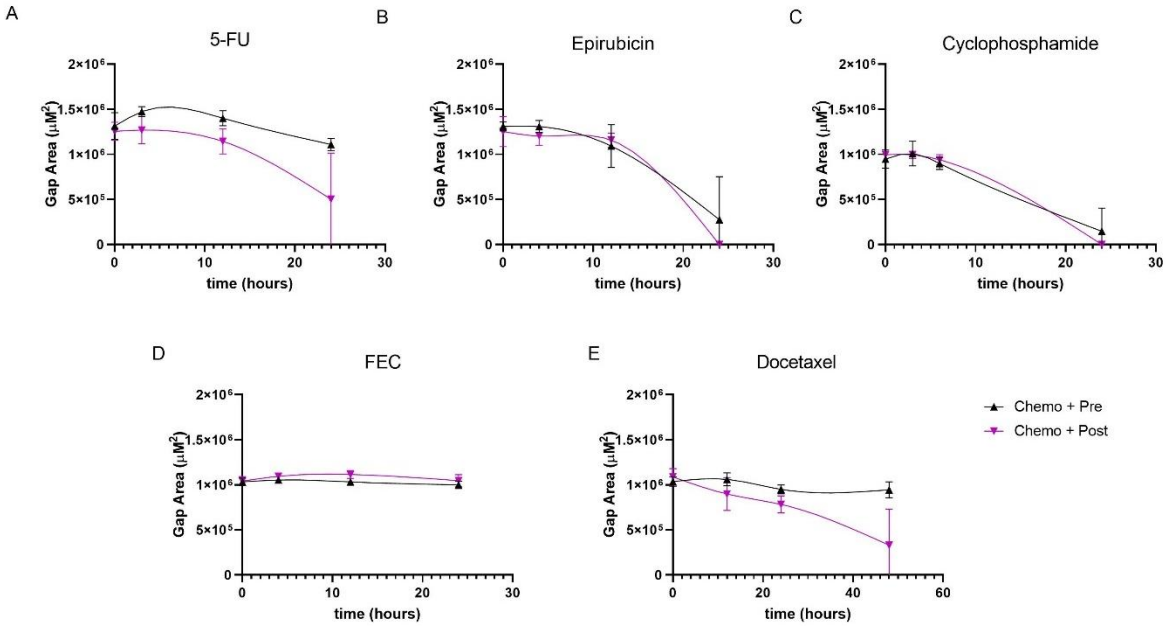

Figure 7.7. HCAEC wound closure (Gap area (μM^2)) over time (hours) with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), FEC combined (D), and docetaxel (E) comparing pre- and post-exercise training intervention serum preconditioning. *Data are presented as Mean ± SEM (n=3).*

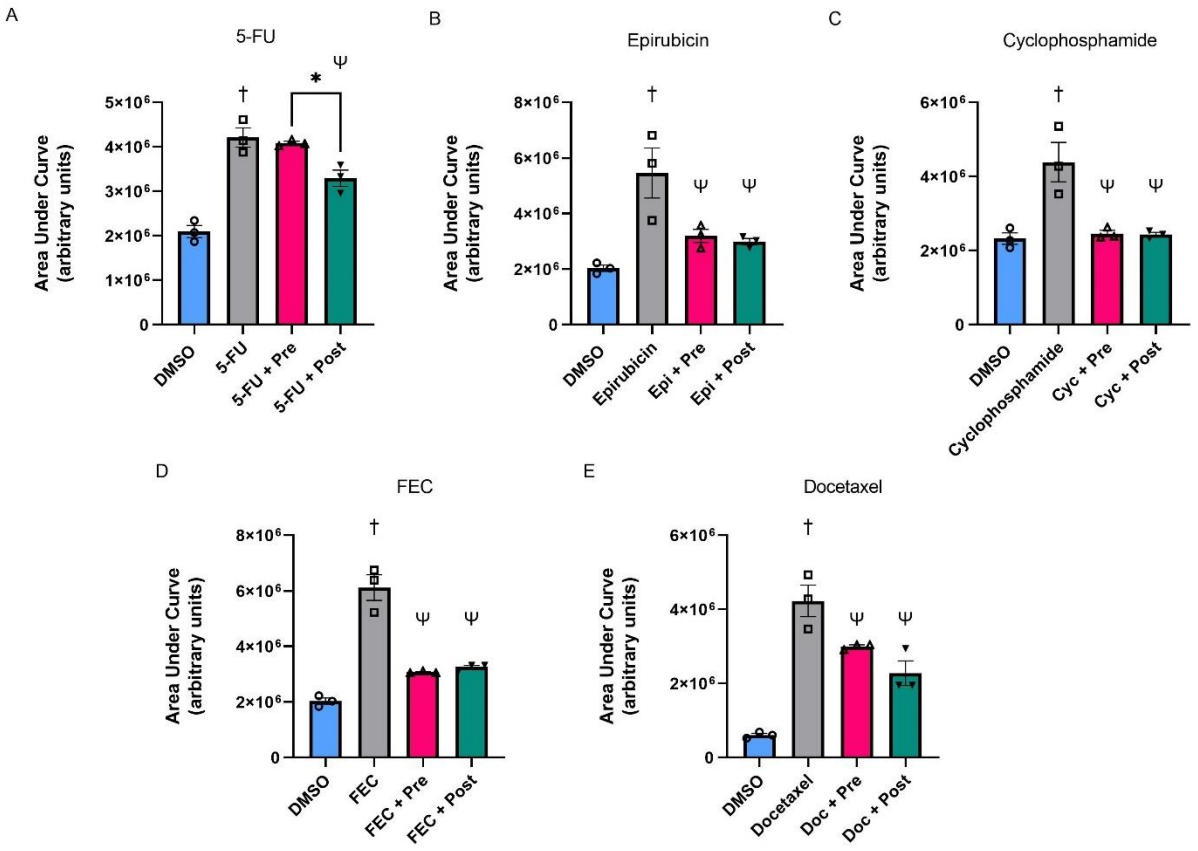

Figure 7.8. Area under the HCAEC gap closure curve with exposure to 5-fluorouracil (A), epirubicin (B), cyclophosphamide (C), FEC combined (D), and docetaxel (E) treatments with DMSO vehicle control, drug with no serum, drug with pre-exercise intervention serum, and drug with post-exercise intervention serum, as determined by ANOVA. *Data shown are mean ± SEM (n=3). *p <0.05 pre- to post-group difference, † significant compared to DMSO, Ψ significant compared to drug only condition.*

7.4 Discussion

The aim of this study was to determine if there is a protective effect of an exercise intervention on vascular toxicity of chemotherapy using an *ex vivo* serum preconditioning approach. Overall, preconditioning endothelial cells with serum collected after an exercise intervention elicits a protective effect against FEC chemotherapy, with a 29% decrease in phosphatidylserine expression and 25% reduction in cleaved caspase-3 expression, compared to pre-training levels **(Figure 7.3A&B)**. This suggests that apoptosis was attenuated with exercise intervention serum. However, there was no effect observed with docetaxel exposure on cell death markers **(Figure 7.3C&D)**. Additionally, wound healing was promoted in the post-exercise serum conditioned group with exposure to 5-FU and docetaxel **(Figure 7.8)**. However, it must be noted that there were no significant differences observed between serological preconditioning groups for eNOS **(Figure 7.4)** and ICAM-1 expression **(Figure 7.5)**. Nonetheless, an exercise intervention may provide some protective benefits for the vascular endothelium, despite no significant changes in BMI, body fat percentage, or blood pressure (**Table 7.1**).

The 'multi-hit' hypothesis suggests that it is not only the chemotherapy itself but a number of factors which contribute to an elevated cardiovascular risk within BC survivors, including pre-existing traditional CVD risk factors (Kirkham, Beaudry, Paterson, Mackey, & Haykowsky, 2019). Hence, it is of interest to investigate protective effects of exercise in individuals who are at risk of developing CVD. The participants in this study are considered 'at risk' of CVD due to presence of hyperglycaemia, hypertension, obesity, and hyperlipidaemia (Dahlöf, 2010). These participants are relevant to BC patients as their characteristics are similar, with 63–65% BC patients having overweight/obesity, 30–33% hypertension, 4% diabetes, and 11% hypercholesterolemia (Park et al., 2017). Hence, this study allows conclusions to be drawn that for those who are already at risk of developing cardiovascular toxicity when exposed to chemotherapy (Kirkham et al., 2019).

The 29% decrease phosphatidylserine expression and 25% reduction in cleaved caspase-3 expression **(Figure 7.3A&B)** suggests that post-exercise intervention serum has modestly attenuated the apoptotic response to FEC exposure in comparison to pre-exercise training serum. This may have implications within a clinical setting. If it is possible to attenuate the chemotherapy toxicity on the vascular endothelium, then this could reduce overall CVD risk

(Sullivan et al., 2000; Verma et al., 2003). As this is a novel approach to investigating exercise effects on vasculo-toxicity, there are no *in vitro* studies of serum preconditioning on endothelial cells exposed to chemotherapy by which to directly compare the current results. However, *in vivo* and *ex vivo* models of exercise interventions prior to doxorubicin exposure found reduced cardiomyocyte oxidative stress and doxorubicin accumulation, responsible for reduced cardiomyocyte apoptosis (Naaktgeboren et al., 2021). It is likely that contributing mechanisms are similar for endothelial cell. Additionally, non-chemotherapy studies can be used to identify potential contributing factors for the observed findings. Exercise training results in lower circulating levels of pro-inflammatory cytokines, including TNF-α, potentially contributing to decreased apoptosis (Henrique et al., 2018; Joussen et al., 2009), as observed with post-exercise serum conditioning **(Figure 7.3A)**. Moreover, reductions in serum glucose levels with exercise training (Slentz et al., 2016) may also have contributed to the reduced endothelial cells apoptosis when exposed to FEC chemotherapy (Risso et al., 2001). The suggested inflammatory and metabolic factors are not exhaustive and were not assessed within this study, providing an interesting area for future mechanistic research. Regardless of the contributing mechanisms, the reduction in endothelial apoptosis with post-exercise training serum when exposed to FEC **(Figure 7.3A)** is a promising finding which adds to current knowledge that an exercise intervention before chemotherapy has potential to alleviate vascular toxicity.

Further comparisons of cardiovascular protection can be drawn from *in human* studies of exercise interventions during and after chemotherapy for BC, with an overall reduction in CVD risk of 20%, occurring alongside a 4.4mmHg reduction in systolic BP and 1.3mmHg reduction in diastolic BP (Lewington et al., 2002; Sturgeon et al., 2014a). More specifically, there has been an 11% decreased risk of developing CVD observed in BC patients participating in a 16-week exercise intervention during chemotherapy, as determined by Framingham Risk Scores (Lee et al., 2019). Additionally, an exercise intervention during chemotherapy prevented the usual increase in carotid intima media thickness in BC patients (Lee et al., 2019b), indicating a reduction in chemotherapy-induced atherosclerotic plaque development. Although not investigated, this suggests a reduction in endothelial damage/dysfunction, as this is the initiating step in atherosclerosis (Cameron et al., 2016b). However, none of these studies directly measured endothelial damage. Furthermore, there

are important differences in our exercise intervention design which differs from these studies, including that these studies are during and after treatment completion. The current study is a pre-habilitative exercise model and, therefore, adds to the literature that there may be exercise protection at an endothelial cell level with an intervention before initiation of chemotherapy and this may be an effect which can reduce CVD risk in BC patients.

So far, exercise interventions to counteract cardiovascular toxicity of chemotherapy have often used $\dot{V}O_{2max}$ as an outcome measure (Scott et al., 2018), finding that exercise can improve cardiorespiratory fitness. However, the assessment of $\dot{V}O_{2max}$ is not specific to the cardiovascular system as muscle tissue also significantly contributes to $\dot{V}O_{2max}$ (Bassett & Howley, 2000). There is a requirement for exercise intervention studies in cancer care to have more direct measures of cardiovascular health and function as currently these studies are scarce (Scott, Adams, Koelwyn, & Jones, 2016). In the current study, $\dot{V}O_{2max}$ showed modest non-significant increase from pre- to post-intervention (**Table 7.1**) but there were still some serological changes which promoted vascular endothelial viability (**Figure 7.3A&B**), adding to knowledge that exercise may still be beneficial to the vasculature, independent of alterations in fitness.

Non-cancer studies of individuals with CVD are useful for drawing further conclusions about the current findings. For example, an exercise intervention reduced coronary artery restriction by >50% in CAD patients (Hambrecht et al., 2000). This is potentially relevant for chemotherapy-induced vascular toxicity and our study adds to the literature by addressing the potential exercise protection of coronary artery endothelial cells against chemotherapy toxicity, as these are the most physiologically relevant endothelial cells for chemotherapy-induced myocardial ischemia and CV event risk, and hence the chosen HCAEC model is perhaps of clinical relevance. The current findings that FEC-induced endothelial damage is alleviated at a cellular level by the systemic environment of serum after an exercise intervention in 'at-risk' individuals adds to the evidence for exercise to counteract pathological vascular states and may contribute to the 20% reduction in CVD risk with exercise training during chemotherapy treatment (Sturgeon et al., 2014a). Exercise training in non-cancer hypertensive patients prevented endothelial damage as evidenced by reduced CEC numbers (Lopes et al., 2018). This is similar to the current findings that exercise serum preconditioning protected against FEC-induced early- and late-apoptosis in HCAECs

(**Figure 7.3A&B**) and perhaps exercise training can precondition the endothelium to a state which evades damage from toxic stimuli.

Despite reduction in endothelial cell deaths, the current study found no effect of the training intervention on eNOS expression for FEC or docetaxel **(Figure 7.4)**. This data is in contrast to previous work where an 8-week HIIT cycling intervention alongside anthracycline-chemotherapy treatment increased brachial FMD by a clinically meaningful 4.3% in the exercise intervention group (Lee et al., 2019b). However, Jones et al. (2013) found no significant change in vascular function from pre-to post-anthracycline chemotherapy between active or sedentary groups, in line with the current findings. More research is required to clarify these contrasting results but perhaps differences in exercise protocols are explanatory. Evidence from animal studies investigating the protective effects of exercise during chemotherapy on vascular function show that 8, but not 4 weeks of exercise training prior to exposure to 5-FU was associated with enhanced endothelium-dependent vasodilation in rats, suggesting that exercise effects need to be of a chronic nature to elicit protective effects on the vasculature (Hayward et al., 2004). However, this does not explain our non-significant results as a 12-week intervention should be sufficient to elicit a protective effect.

Moreover, exercise interventions have been shown to improve vascular endothelial health and function in clinical populations, including that CAD patients who underwent a 4-week exercise training programme before surgery had a 57% enhanced endothelial function, compared to those who did not exercise. Exercise-trained patients also exhibited a 2-fold higher eNOS protein expression and 4-fold higher eNOS activation levels, compared to sedentary controls, suggesting that exercise has improved endothelial health (Hambrecht et al., 2003). The higher eNOS content and activation, together with enhanced vascular relaxation, indicates strong protective effect of exercise in at-risk populations. Differences between *in-vivo* studies and the current study is that exercise-induced protection of endothelial function *in vivo* may not be due to systemic serum adaptations to exercise. The increased endothelial function seen in some exercise studies perhaps require exercise-induced increases in shear stress to induce adaptations in endothelial function and health (Gielen, Sandri, Erbs, & Adams, 2011), as eNOS is produced upon mechanical stimulation of the endothelium (Balligand, Feron, & Dessy, 2009). During exercise, blood flow is increased,

and this may be responsible for increases in eNOS production with exercise training (Balligand et al., 2009). As shear stress has not been utilised in this cell culture model, it is not possible to provide a conclusion on this. It may be the case that serological factors alone cannot induce endothelial protection, and this may explain the difference between findings *in vitro* and *in vivo*.

As well as no change in endothelial eNOS content, there was no difference in HCAEC ICAM-1 expression from pre- to post-exercise intervention for FEC or docetaxel **(Figure 5)**. There are no other known *in vitro/ex vivo* studies of exercise serological preconditioning on chemotherapy toxicity on endothelial cells by which to compare these findings. Therefore, these results are novel and add to the literature. However, further investigation is required for confirmation and exploration of both consequence and mechanism. Interestingly, a recent review of the effects of exercise on adhesion molecule expression in humans (non-cancer) found that exercise elicited either no change or a decrease in soluble adhesion molecules across a wide range of 'at risk' populations (Palmefors et al., 2014b). Both obese and normal-weight adolescents decreased soluble ICAM-1 after 8-week moderate-intensity endurance or interval training. Likewise, CVD patients reduced soluble ICAM-1 with 8-12 weeks of moderate-intensity exercise (Koh & Park, 2018). As with eNOS regulation, the downregulation of adhesion molecules with exercise may not be due to serological factors. The main mediator of exercise-induced regulation of adhesion molecules has also been determined to be shear stress (Tinken et al., 2010) and therefore, a shear stress stimulus may be required to elicit this response *in vitro*. Since no other *in vitro* study has investigated the effects of serological preconditioning of cells with exercise serum on adhesion molecule expression, it is not possible to draw definite conclusions. Perhaps the addition of chemotherapy reverses the protective effects of exercise serum preconditioning. Currently, there are no *in-human* studies investigating effects of exercise before chemotherapy on adhesion molecule expression. Therefore, this is the first ever study investigating effects of an exercise intervention, chemotherapy, and endothelial adhesion molecules. The current study shows that an exercise intervention does not attenuate the effect of FEC-T drugs on the expression of adhesion molecules **(Figure 7.5)** and that serological protection of endothelial cells is independent of ICAM-1 regulation.

Serum preconditioning after an exercise intervention elicited significant protective effects on endothelial wound repair when exposed to 5-FU and docetaxel, shown in **Figure 7.6A & 7E**. Currently, there are no known studies of endothelial wound healing in presence of chemotherapy and serum from an exercise intervention in which to compare the current findings. However, the exercise-induced protection against 5-FU and docetaxel wound healing impairment may be due to enhanced growth factors in the serum, specifically VEGF (Grunewald et al., 2021). This is concurrent with the literature as an 8-week exercise intervention increased skeletal muscle VEGF levels in heart failure patients, associating with improved skeletal muscle angiogenesis (Gustafsson et al., 2001). VEGF promotes longevity in mice, through inhibition of physiological aging across the whole cardiovascular system. In mice, improving VEGF signalling preserved capillarisation, improved organ perfusion and function, reducing cardiovascular risk and extended life span (Grunewald et al., 2021).

Furthermore, exercise interventions have been shown to increase circulating HDL-cholesterol (Palazón-Bru et al., 2021). This could be a mediating mechanism behind the promotion of wound healing in 5-FU and docetaxel conditions, as HDL-cholesterol stimulates endothelial cell migration for wound repair in HCAECs (Kimura et al., 2003). This is relevant to *in vivo* animal model studies as HDL promotes wound healing in aged mice, likely through increasing VEGF (Tsatralis et al., 2016). This highlights a potential role for HDL in the therapeutic modulation of vascular complications and is a potential mechanism behind the exercise-induced cell migration-proliferation observed with 5-FU and docetaxel drug conditions. Interestingly, HDL can protect cardiomyocytes against doxorubicin toxicity in cardiomyocytes via anti-apoptotic pathways (Durham, Chathely, Mak, et al., 2018; Durham, Chathely, & Trigatti, 2018; Durham et al., 2019; Kimura et al., 2003), and hence, HDL may protect against both apoptosis and wound repair efficiency. Now, for the first time, the current findings have added to this knowledge that serum preconditioning post-exercise intervention is also protective against vascular endothelial health and this may also be via HDL and VEGF modulation. This said, there are several other potential exercise-induced changes within human serum which may be contributing to the protection of endothelial cells, including attenuations in metabolic (Kruse, Singh, Sørensen, Eriksson, & Nuutila, 2016; Slentz et al., 2016), hormonal (Pedersen et al., 2016; Stojadinovic, Gordon, Lebrun, & Tomic-Canic, 2012), anti- and pro-inflammatory factors (Eming, Krieg, & Davidson,

2007; Petersen et al., 2011). Nonetheless, improvements in vascular health and function coupled with improvements in cardiac health will help to protect against the chemotherapy cardiovascular toxicity and help to improve disease-free survival, promoting longevity and quality of life.

7.5 Conclusions and Implications

To conclude, this study has found that preconditioning endothelial cells with serum after a 12-week HIIT intervention has significantly protective effects on indicative markers of FEC-induced apoptosis, although does not offer protection against docetaxel exposure. There is also no significant effect of endothelial cell preconditioning with post-exercise intervention serum on endothelial cell function as measured by eNOS expression and adhesion molecule expression. Significant findings did not occur for every outcome measure within every drug condition and therefore, must be considered with the perspective that cardiovascular toxicity is multi-faceted, complex, and interactive. Although hopeful that findings will be relevant to *in human* studies and that comparisons can be drawn, findings are in fact exclusively *in vitro* and must be considered with modesty. Nonetheless, a reduction in endothelial cell death is promising, and this model mimics exercise 'prehabilitation' for those undergoing chemotherapy treatment to precondition the vasculature to an anti-apoptotic state. Overall, this study adds to the growing evidence to emphasise the need for an exercise intervention before chemotherapy for BC to prevent CV toxicity as this could help to reduce additional preventative treatment costs and future CV management and treatment costs. An exercise intervention could be implemented into BC care between diagnosis and receiving chemotherapy to reduce future CV events, without incurring the high cost or potential detriments of pharmaceutical alternatives.

Chapter 8

General Discussion

8.1 Main Findings

The aim of this thesis was to investigate the effects of chemotherapy on endothelial cells, and to assess the effects of physical activity/exercise on the vascular toxicity of chemotherapy using an *ex vivo* model. Many novel findings have resulted from the studies included in this thesis. These main findings are:

Aim 1: To determine the dose-response effects of physiological concentrations of FEC-T chemotherapy on endothelial cells (*Chapter 4*).

Main Findings:

(1) There is a strong effect for all FEC-T drugs individually and as combined FEC treatment on reducing endothelial cell viability and inducing apoptosis, as determined by cleaved caspase-3 and phosphatidylserine expression.
(2) There is a strong dose-dependent response for docetaxel and FEC combined treatment on reducing endothelial cell eNOS content.
(3) FEC-T drugs also reduce endothelial adhesion molecule expression.
(4) FEC-T drugs individually, and as combined FEC treatment, inhibit endothelial wound repair.

Hypothesis: FEC-T chemotherapy induced vascular endothelial toxicity in a dose-dependent manner through inducing apoptosis (*Accept*), reducing eNOS content (*Accept*), increasing adhesion molecule expression (*Reject*), and inhibiting wound repair (*Accept*).

Aim 2: To determine the effect of human serum preconditioning on the endothelial cell response to FEC-T exposure, comparing serum from physically active and inactive females (*Chapter 5*).

Main Findings:

(1) There was significantly reduced cell death in endothelial cells preconditioned with serum from active compared inactive individuals in endothelial cells exposed to 5-FU and epirubicin in HUVECs and docetaxel in HCAECs.
(2) There was significantly higher eNOS content by preconditioning with serum from active compared to inactive individuals in HCAECs when exposed to docetaxel.

(3) Endothelial adhesion molecule expression was preserved in the active vs inactive conditioning group with FEC and docetaxel exposure.
(4) Preconditioning with serum from active compared to inactive individuals negated the impacted of FEC-T chemotherapy on endothelial wound repair.

Hypothesis: serum from physically active women offers protection against chemotherapy-induced vascular endothelial cell toxicity, when compared to control serum from inactive women (*Accept*).

Aim 3: To determine the potentially protective effects of a single exercise session prior to chemotherapy exposure on vascular endothelial cells (*Chapter 6*).

Main Findings:

(1) Serum preconditioning after acute exercise offers significant vascular endothelial protection from FEC-induced cell death but not docetaxel-induced cell death, as compared to serum collected before the exercise session.
(2) Serum preconditioning after acute exercise had no effect on endothelial eNOS expression or wound repair when exposed to any of the FEC-T drug conditions, compared to pre-exercise serum.
(3) Serum preconditioning after acute exercise attenuates the usual decrease in endothelial cell ICAM-1 expression with docetaxel exposure to a significantly greater extent than serum collected before exercise.

Hypothesis: serum collected after an acute exercise session can alleviate vascular endothelial cell toxicity of FEC-T treatment, compared to serum collected before an acute exercise session (*Accept*).

Aim 4: To determine the potentially protective effects of serum collected after an exercise training intervention on the effects of chemotherapy exposure on vascular endothelial cells (*Chapter 7*).

Main Findings:

(1) Preconditioning endothelial cells with serum taken after an exercise training intervention elicits a protective effect against cell death induced by FEC chemotherapy but has no effect against death induced by docetaxel exposure.

(2) There was no within-group difference for pre- and post-exercise intervention serum preconditioning on endothelial cell eNOS expression or ICAM-1 expression when exposed to FEC-T drugs.
(3) Preconditioning endothelial cells with post-exercise intervention serum alleviates impairment of endothelial cell wound repair with 5-FU and docetaxel exposure, as compared to pre-exercise intervention serum.

Hypothesis: serum obtained after an exercise intervention can alleviate endothelial cell toxicity of FEC-T chemotherapy exposure even in those previously untrained even in those 'at risk' for cardiovascular events (*Accept*).

8.2 FEC-T Chemotherapy Induces Endothelial Cell Apoptosis, eNOS Downregulation and Impaired Wound Repair Ability

Firstly, to determine the toxic effects of FEC-T chemotherapy commonly used in early-BC treatment, an endothelial cell model of chemotherapy exposure was developed. The current findings provide strong evidence that FEC-T drugs individually and FEC combined induce significant vascular endothelial toxicity *in vitro* in both HUVEC and HCAEC models of chemotherapy exposure, as demonstrated by reduced endothelial cell viability, occurring alongside increased expression of phosphatidylserine and cleaved casoase-3, indicating cell death by apoptosis. These findings are consistent to the literature as other *in vitro* studies of the effects of chemotherapy exposure on endothelial cells have also found significant endothelial apoptosis upon chemotherapy exposure (Altieri et al., 2017; Bocci, Nicolaou, & Kerbel, 2002; Bosman et al., 2021; Eakin et al., 2020; S. Wu, 2002; Yamada et al., 2012). It is likely that endothelial apoptosis occurs via the intrinsic apoptotic pathway as FEC-T drugs damage endothelial cell DNA and RNA (Longley et al., 2003), inhibit DNA synthesis via tubulin stabilisation (Field et al., 2015)and topoisomerase inhibition (Stěrba et al., 2013), and cause mitochondrial damage through generation of ROS (Florescu et al., 2013; Stěrba et al., 2013). These apoptotic stimuli signal activation of p-53 gene which initiates the caspase cascade, leading to cleavage of cellular components, resulting in apoptosis and phagocytosis of the apoptotic cell via macrophages (Elmore, 2007; Lawen, 2003).

These *in vitro* findings bear relevance *in vivo*, as chemotherapy increased CECs by 3-fold in BC patients after just a single dose, indicating acute endothelial injury (Beerepoot et al., 2004). This reduction in endothelial cell viability has implications for vascular health, with endothelial insult without efficient repair being one of the mediating mechanisms behind the initiation of vascular disease and CVD mortality (Werner et al., 2008). This may well be a contributing factor towards the cardiovascular toxicity of chemotherapy, which is attributed to 15.9% of deaths in BC survivors (Patnaik et al., 2011a). Toxicity also occurs within cardiac cells and this is well-established to contribute to CVD observed in cancer survivors (Cardinale et al., 2002b; Malik et al., 2016). Understanding the mechanisms behind CVD development in BC survivors is important for treatment management and prevention of CVD. Now, endothelial damage and dysfunction from chemotherapy may be included as a contributing or initiating factor in toxicity as vascular damage is strongly linked to initiation of CVD (Verma et al., 2003). Findings

that chemotherapy also damages vascular endothelial cells adds to the pool of evidence that chemotherapy is toxic to the whole cardiovascular system, and therefore, should be considered as a whole system when addressing chemotherapy cardiovascular toxicity. Damage to cardiac and vascular endothelial cells has devastating consequences for the cardiovascular system, which may ultimately lead to heart failure and premature death, as depicted in **Figure 8.3**.

The current findings that chemotherapy not only causes endothelial cell death but also dysfunction as illustrated by a reduction in eNOS expression, also agrees with the literature (Gajalakshmi et al., 2013; Sekijima et al., 2011) and is of potential clinical importance for cardiovascular toxicity in cancer survivors. A reduction in eNOS expression leads to reduced NO production and release, thus vasodilatory capacity is reduced, resulting in systemic hypertension, increased risk of thrombosis (Antonella De Angelis et al., 2017) and CVD (Benjamin et al., 2004). Relevant *in human* studies have observed a reduction in vascular function (as measured by FMD) with chemotherapy treatment both immediately (Duquaine et al., 2003a) and longer-term (Vassilakopoulou et al., 2010). FMD is predictive of chemotherapy cardiotoxicity, with every 2.7% increase in FMD associating with a 37% less likelihood for LVEF reduction at 3 months post-chemotherapy treatment in BC patients (Anastasiou et al., 2017). Hence, reduced vasodilatory capacity and thrombosis risk is likely a mechanism contributing to the 5% incidence of HF associated with chemotherapy (Sandra M. Swain et al., 2003b). The reduced eNOS content leads to thrombosis (Hansson, 2005), vasospasm and endothelial dysfunction, resulting in vasoconstriction, leading to hypertension (Hansson, 2005) and ischaemia (Pai & Nahata, 2000), increasing the risk for MI (Pai & Nahata, 2000) and strain on the heart leading to a hypertrophic myocardium, cardiac dysfunction and potentially heart failure (Brower et al., 2006) (**Figure 8.3**).

Additionally, results show that chemotherapy consistently downregulated ICAM-1 despite the expectation of increased stimulation of ICAM-1 expression due to endothelial activation as shown previously with other chemtoherapy and endothelial cell studies (Meijer, 2009). Although these results differ to the hypothesis, the flow cytometric method was validated **Appendix 8** using TNF-α to promote adhesion molecule expression in these endothelial cells. The difference in our results is yet to be fully explained but this may be due to the different incubation times (Meijer, 2009) or location of protein analysis (cell surface vs. intracellular)

(Volanti et al., 2004). Meijer *et al*. (2009) anlaysed endothelial cells at 24-, 48- and 72-hour time points after exposure to cisplatin (alyklating agent similar to cyclophosphamide). The time-point in the current studies was 4-hours for FEC exposure and hence, this may be an effect which occurs onwards of the 4 hour time-point utilsed in the current anaysis. The effect of ICAM-1 expression in response to chemotherapy exposure (as measured by flow cytometry) over a 72-hour period is illustrated in **Figure 8.1**. The experiment should therefore be repeated using multiple time-points over a 72-hour period to determine time-dependent effects. It may well be the case that endothelial cell adhesion molecules are initially downregulated before upregulation. There is evidence for this reasoning in *in vivo* work, as soluble ICAM-1 is initially downregulated with chemotherapy exposure (at cycle 2) before being elevated at cycle 4 (Mills et al., 2004). This response over time is depicted in **Figure 8.1**. This suggests that repeated exposure would be required to initiate this response *in vitro*. Therefore, this creates scope for the experiment to be repeated using repeat exposures of FEC-T. Furthermore, in a non-chemotherapy study of photodynamic therapy (use of light sensitive medicine and a light source to destroy abnormal cells), endothelial ICAM-1 expression is upregulated at a transcriptional level but is downregulated at a protein level due to degradation by lysosomal proteases (Volanti et al., 2004). This process cannot be ruled out as an explanation for the findings observed within the current study. This effect may also be observed with apoptotic blebbing due to chemotherapy exposure as this also downregulates surface marker expression despite the upregulation of transcription (Navratil et al., 2001). To confirm or reject this hypothesis, the experiment must be repeated utilising further analysis techniques including PCR to determine effects at a transcriptional level. Therefore, this PhD provides an initial step for confirmatory studies which explore more in depth the effects of chemotherapy on endothelial cell adhesion molecules both at a transcriptional and protein level.

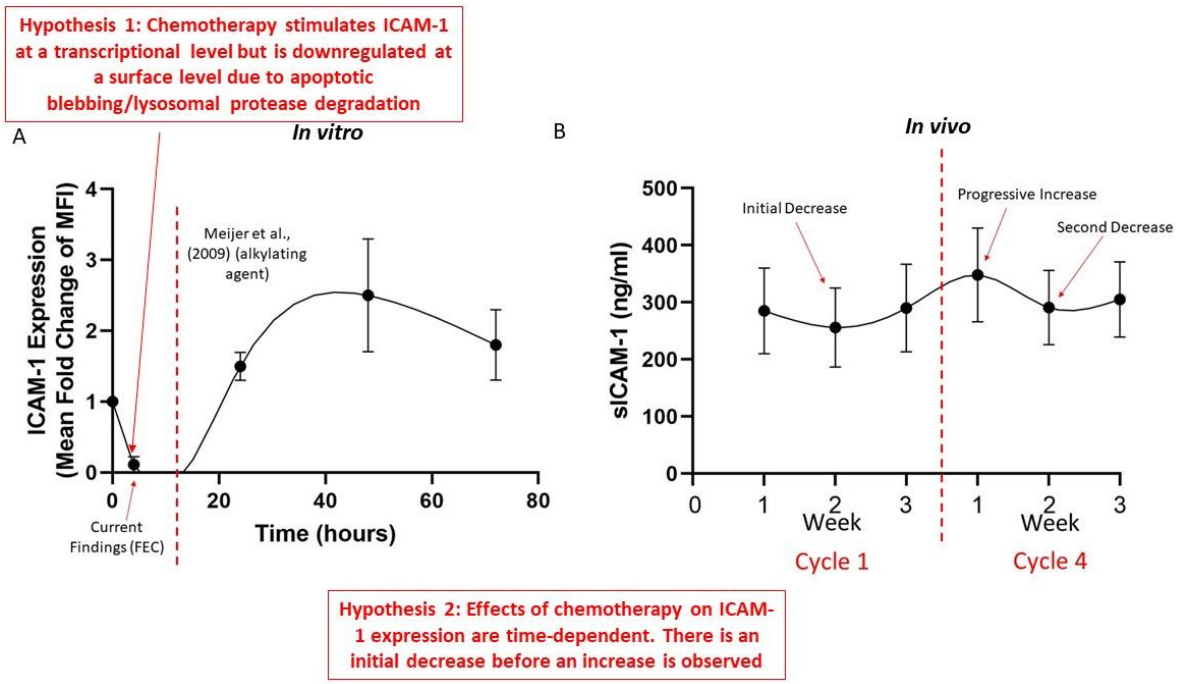

Figure 8.1. The current study findings of the effects of FEC exposure on endothelial cell ICAM-1 protein expression *in vitro* at 4 hours, displayed with current literature knowledge of endothelial cell ICAM-1 expression at 24-72-hours post-exposure *in vitro* (Meijer et al., 2009) (A); and current literature knowledge of soluble ICAM-1 expression over time in BC patients undergoing chemotherapy cycles (B: Mills et al., 2004). *Data displayed are Mean ± SD.*

Endothelial repair is also important for reducing CVD risk and the endothelial cells ability to do so is impaired with chemotherapy. Vascular damage withour repair is an initiating step for atherosclerosis and hence increases the risk of CVD (Cameron et al., 2016b; Verma et al., 2003). From our findings, endothelial wound repair is not only inhibited but indeed worsened with some of the chemotherapy conditions. This is illustrated in **Figure 8.2**. Therefore, if this translates to *in vivo* models, it would be expected that any underlying vascular damage would in fact be worsened due to an increase in endothelial apoptosis in the wound assay. This could be a disastrous consequence for initiation of atherosclerosis as wounds in the vasculature have the potenial to accumulate large plaques (Sullivan et al., 2000) and may explain the increased incidence of thrombosis in BC survivors (Haddad & Greeno, 2006). Of course, increased risk of atherosclerosis due to inefficeint wound repair combined with increased wound size, will contribute to overall cardiovascular detirmnets including hypertension and plaque rupture leading to potential MI, ischaemia, and/or HF **(Figure 8.3)**. Vascular wound repair in response to chemotherapy has never before been investigated and therefore, this

is a new finding which can be added to the literature to strengthen evidence for the detrimental effects of chemotherapy on the cardiovascular system.

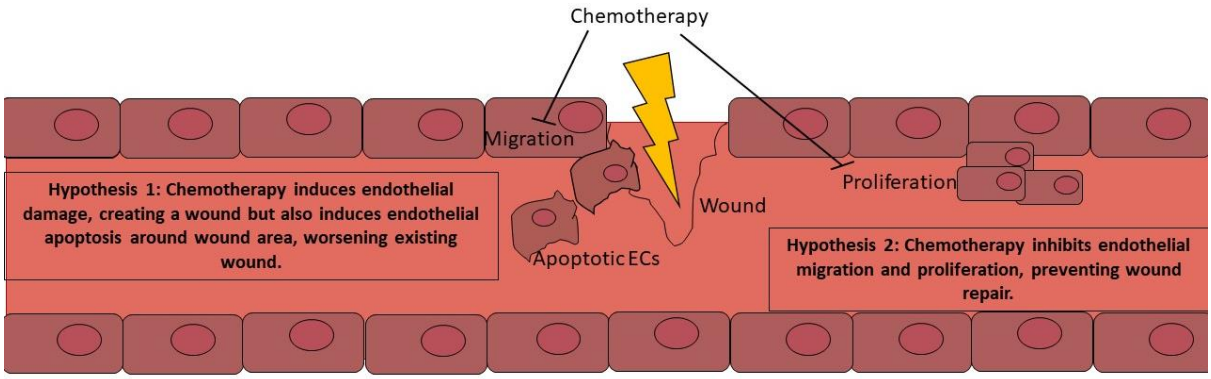

Figure 8.2. Endothelial wound repair is inhibited by chemotherapy-induced damage, endothelial apoptosis, and inhibition of proliferation and migration. *Endothelial cells (ECs).*

Overall, exposure to FEC-T drugs is damaging to endothelial cells, causing cellular death and dysfunction, which leads to vascular detriments including vasoconstriction, atherosclerosis, and vasospasm, resulting in hypertension and myocardial ischaemia. This coupled with direct cardiac toxicity from chemotherapy has potential to worsen risk for MI, cardiac hypertrophy and ultimately lifethreatening heart failure. This process is outlined in **Figure 8.3**.

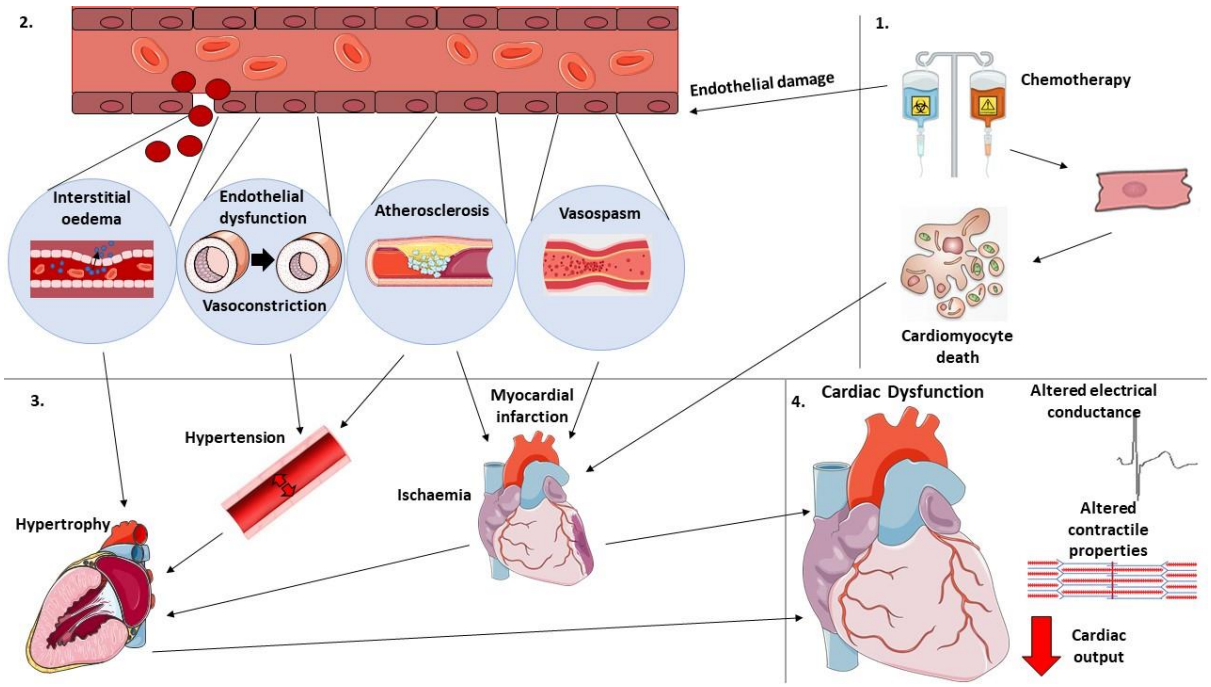

Figure 8.3. Potential mechanisms by which chemotherapy induces cardiac dysfunction via endothelial and cardiomyocyte damage.

8.3 Serological Factors from Habitually Physically Active Individuals Attenuates FEC-T Induced Endothelial Cell Toxicity

There is now emerging evidence for the benefits of physical activity in reducing cardiotoxicity risk in those treated with chemotherapy for BC (Nagy et al., 2017b; Palomo et al., 2017). Therefore, the first step was to investigate the effects of habitual physical activity on vascular toxicity using an *ex vivo* model. The main findings from this study are that there was significantly reduced apoptosis in endothelial cells preconditioned with active serum compared to inactive serum in endothelial cells exposed to 5-FU and epirubicin in HUVECs and docetaxel in HCAECs. However, there was no effect of serum preconditioning on any of the other chemotherapy conditions (cyclophosphamide or FEC combined) for cleaved caspase-3 or phosphatidylserine expression and therefore, this effect must be considered specific to 5-FU and epirubicin in HUVECs and docetaxel in HCAECs. Vascular endothelial cell protection against markers indicative of apoptosis with physically active serum has been previously established in studies of oxidative stress, likely attributed to enhanced serum antioxidants, such as superoxide dismutase and glutathione peroxidase (Bouzid et al., 2018; Conti et al., 2012a). As epirubicin and docetaxel both induce markers indicative of apoptosis via oxidative stress pathways (Florescu et al., 2013; Stěrba et al., 2013), the reduction in apoptosis may well be attributed to increased anti-oxidants in physically active serum, potentially reducing oxidative-stress-induced apoptosis. Therefore, habitual physical activity may well protect against endothelial cell apoptosis by counteracting the mechanism of action of epirubicin and docetaxel. However, further studies investigating oxidative stress are required to confirm this mechanistic link. Nonetheless, attenuation of endothelial apoptosis may play an important role in reducing overall cardiovascular toxicity risk in BC patients. Chemotherapy increases CECs by 3-fold in BC patients after just a single dose (Beerepoot et al., 2004) and this relates to increased vascular disease (Makin et al., 2004). As endothelial insult is one of the mediating mechanisms behind the initiation of CVD (Werner et al., 2008) – as depicted in **Figure 8.3** – reducing endothelial cell death with physical activity will reduce overall cardiovascular toxicity with chemotherapy. This may help to explain results from observational studies of physical activity which have shown higher physical activity levels before and during chemotherapy associates with a 31% and 47% lower risk of CAD and any CV event death, respectively, after a median follow-up of

12.7 years (Palomo et al., 2017). The current results are the first to show this *in vitro* and larger *in vivo* studies are now required to confirm and expand these findings. Furthermore, it must be noted that the composition of serum was not explored within the scope of this PhD and therefore, the mechanism behind sera protection is not yet known. However, it is likely that enhanced serum antioxidants play a role by reducing oxidative stress-induced endothelial cell apoptosis (Bouzid et al., 2018; Conti et al., 2012a). This is likely to occur alongside alterations in anti-inflammatory mediators such as interleukin-1 and -10 and reductions in pro-inflammatory cytokines such as TNF-α (Petersen & Pedersen, 2005), which would reduce inflammatory-mediated extrinsic apoptotic signalling (Joussen et al., 2009). Furthermore, metabolic factors, including improved glucose levels are likely to contribute to reduced apoptosis (Leskinen et al., 2013; Risso et al., 2001). Future investigation to identify potential contributing factors to physical activity protection may uncover potential targets for therapies to attenuate toxicity of chemotherapy treatment in BC patients.

As endothelial dysfunction has been identified as a key contributing factor to CVD (Mudau, Genis, Lochner, & Strijdom, 2012b), the effects of habitual physical activity on endothelial function have also been investigated. In HCAECs only, there was a significantly protective effect of active serum on endothelial eNOS expression with exposure to docetaxel, with no effect on eNOS expression with FEC exposure. As active serum preconditioning of HCAECs also attenuated the apoptotic response to docetaxel but not to FEC, these findings suggest a potential link between eNOS and apoptosis. This is concurrent with the literature as NO production is inhibited in apoptotic endothelial cells (Sato et al., 1993). Physically active serum preconditioning has been shown to increase endothelial NO production in non-chemotherapy studies, also alongside reductions in endothelial apoptosis (Conti et al., 2012a). From this and our own findings, prevention of apoptotic signalling may be required to prevent downregulation of eNOS production or *vice versa.* This is a potential pathway of protection from chemotherapy cardiovascular toxicity via habitual physical activity. However, further *in vitro* experiments are required to explore this potential mechanism, including blocking eNOS activity to remove the potential for NO to inhibit apoptosis. This is potentially important for BC patients as bioavailability of eNOS and subsequent production of NO is critical for preservation of cardiovascular health and may attenuate treatment toxicity. NO acts as a potent vasodilator essential for blood pressure regulation; and elicits

anti-thrombotic and anti-platelet properties (Verhamme et al., 2016). Taken together, these characteristics make NO production crucial to prevent hypertension and atherosclerosis development, ultimately protecting against cardiovascular events and HF (Mudau et al., 2012b). Additionally, an increase in NO production may also protect against autonomic dysfunction observed with chemotherapy exposure for early BC, protecting against cardiac dysfunction (J. M. Scott et al., 2014). Interestingly, physical activity associates with higher eNOS and NO production as measured by FMD in non-cancer populations (Pahkala et al., 2011). This is thought to be due to chronically increased shear stress along the endothelium which stimulates eNOS to produce NO (Wedgwood & Black, 2005). For the first time, the current study has shown that there is contribution of systemic changes in the circulation of physically active females which also results in increased eNOS expression. One potential systemic change with physical activity could be increased anti-oxidants, reducing oxidative stress (Conti et al., 2012a). Again, this has not been explored within the scope of this thesis and requires further investigation. Nevertheless, the role of physical activity in regulating endothelial NO production may be key for protection against chemotherapy-associated cardiovascular toxicity, and this has been shown for the first time *in vitro*. However, it must be noted that there was no effect of physically active serum preconditioning on endothelial eNOS expression for FEC combined drugs. Therefore, the chosen chemotherapy treatment regimen plays an important role in whether habitual physical activity is effective in preventing cardiovascular toxicity. This should be explored further by repeating experiments with different chemotherapy drugs and common combinations used within BC care (such as doxorubicin, methotrexate, and paclitaxel (Anampa, Makower, & Sparano, 2015)). Determining which particular chemotherapy regimens benefit from regular physical activity will provide recommendations for patient care. As this is the first study of its kind, the development of the serological model opens the possibility for further exploration of the effects of physical activity on vascular endothelial toxicity with other relevant chemotherapeutics.

The effects of *ex vivo* serum preconditioning before endothelial exposure to FEC-T drugs on adhesion molecule expression was also investigated but results create more questions for future research. Serum from active females resulted in significantly higher adhesion molecule expression compared with serum from inactive females. Increased adhesion

molecule expression is indicative of endothelial activation which is a state induced by physiological stress (Liao, 2013). It was expected that physically active serum would decrease adhesion molecule expression, as shown *in vivo* (Koh & Park, 2018). However, the opposite effect was the case. This data can be interpreted with confidence as the assay was thoroughly validated in **Appendix 8**. A possible explanation for the current finding is that serum preconditioning attempts to restore endothelial cell homeostasis to counteract the chemotherapy-induced downregulation of ICAM-1 surface expression. This may well occur via reduction in endothelial apoptosis (also evident in *Chapter 5*), as this may diminish the apoptotic membrane shedding and subsequently decreased endothelial cell surface expression of ICAM-1 (Navratil et al., 2001). The potential mechanism suggested is illustrated in **Figure 8.4**. There are no other known studies of exercise serological preconditioning on chemotherapy toxicity on endothelial cell adhesion molecule expression by which to compare these findings. Therefore, these results are the initial step in investigation of endothelial adhesion molecules in exercise oncology and further investigation is required for exploration of both consequence and mechanism. Future studies should include confirmatory data with active and inactive females, and progress to using serum from different populations, including BC females of different ages participating in specific types and durations of physical activity. To determine the consequence of effects of physical activity on adhesion molecule expression after exposure to chemotherapy, more complex experiments are required, including studies of leukocyte adhesion to the endothelium. This may well be explored by utilising the endothelial cell conditioning model developed in this study as the basis for future research.

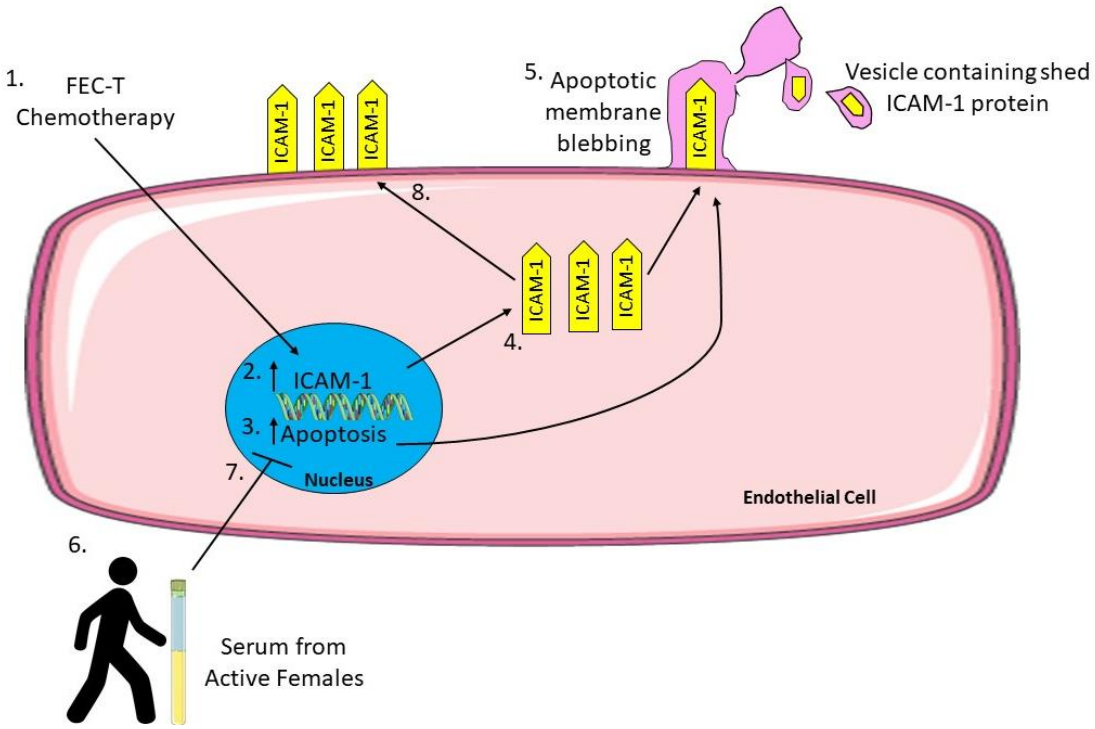

Figure 8.4. FEC-T chemotherapy (1) inducing upregulation of endothelial cell ICAM-1 transcription (2) and induction of apoptosis (3), with ICAM-1 protein transportation (4) to the endothelial cell surface where apoptotic membrane blebbing (5) creates vesicles containing the ICAM-1 protein, preventing endothelial cell surface ICAM-1 expression. Serum from active females (6) inhibits apoptosis (7), allowing ICAM-1 to be expressed on the endothelial cell surface (8).

Furthermore, in both HUVECs and HCAECs, physically active individuals' serum provided protection against FEC combined and docetaxel wound repair disruption. Similar results were found in non-chemotherapy studies whereby habitually active serum had a pro-proliferative effect on HUVECs (Conti et al., 2012b; Sapp et al., 2020). This is associated with enhanced VEGF (Sapp et al., 2020) and NO content (Conti et al., 2012b). This is in line with our study findings that wound repair was preserved alongside preserved eNOS expression. Therefore, NO may well be required for efficient endothelial cell wound repair when exposed to docetaxel, and both these functions are preserved with active vs inactive serum preconditioning. The use of an eNOS blocker/donor in future studies will provide confirmation of the role of NO in wound repair with exercise during chemotherapy. Furthermore, the VEGF content of the serum was not analysed and hence, future studies should investigate this and determine if this is a contributing factor to enhance wound repair with experimental addition of a VEGF-blocker. These findings are potentially

important for BC patients as these could be future therapeutic targets for toxicity prevention. It is important to note that active serum's attenuation of apoptosis in response to docetaxel exposure may play a role in defending against wound repair disruption. Wounds which did not repair (and even worsened over time) had the appearance of apoptotic cells along the gap edges, and in some cases, dead cells broke away from the gap edge and increased the size of the wound. However, preconditioning endothelial monolayers with serum from active individuals prior to docetaxel exposure prevented the increase in size of the wound area, allowing the wound to fully repair over time. This may be due to prevention of docetaxel-induced apoptosis and preservation of eNOS expression with active serum preconditioning, as observed in flow cytometry analysis. The effect of active serum preconditioning with docetaxel exposure on endothelial wound repair is illustrated in **Figure 8.5**. This has potential importance *in vivo* as physical activity may prevent docetaxel exposure from further damaging any underlying scratches/wounds present throughout the cardiovascular system of cancer patients. Ultimately, this may help to protect against atherosclerosis in those undergoing chemotherapy treatment, as endothelial damage without repair is a mechanism underpinning atherosclerosis initiation and progression (Cameron et al., 2016b). Hence, protection of the endothelium ability to efficiently repair vascular wounds would protect against cardiovascular toxicity of chemotherapy and this is a promising finding for those undergoing docetaxel regimens.

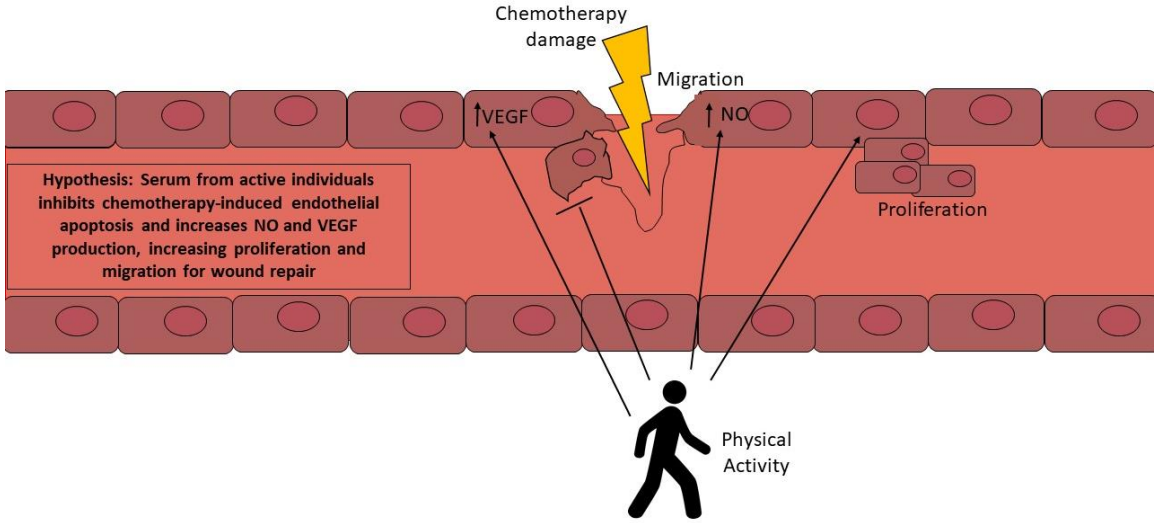

Figure 8.5. The effects of serum from active individuals on endothelial wound repair when exposed to docetaxel chemotherapy.

The overall conclusion from this study is that there may be a beneficial effect of habitual physical activity on endothelial cell toxicity of FEC-T chemotherapy exposure on apoptosis. This is the first study of its kind utilising *ex vivo* serum preconditioning to investigate effects of physical activity on chemotherapy toxicity, and therefore is a starting point for further investigation of exercise in vascular oncology research. Development of this *ex vivo* model provides opportunity to further expand research on exercise and chemotherapy at a cellular level, including investigation of the role of eNOS, VEGF, and antioxidants.

8.4 Serological Conditioning with Serum Collected After Acute Exercise Attenuates Endothelial Cell Toxicity of FEC-T Drugs

Acute exercise serum preconditioning offers significant vascular endothelial protection from FEC exposure, as shown by reduced endothelial apoptosis with serum preconditioning post-exercise compared with pre-exercise serum. This is important for maintaining integrity of the endothelial layer upon exposure to chemotherapy, as this is important in protecting against atherosclerosis plaque development and subsequent cardiovascular detriments, including hypertension, stroke, cardiac ischaemia, myocardial infarction, and heart failure (Brower et al., 2006; Hansson, 2005; Pai & Nahata, 2000). However, there is no protection offered from acute exercise before docetaxel exposure and hence, results are specific to FEC as a combined treatment only. Interestingly, a non-cancer *in vitro* study found that acute exercise serum preconditioning protected against apoptosis in HCAECs, attributed to by increased uptake of EMPs (Wahl et al., 2014). Other *in vitro* studies using serum preconditioning for oncological research found that acute exercise serum preconditioning reduces cancer cell growth by ~9% (Metcalfe et al., 2021; Orange et al., 2020). As the current study is the first to utilise this same *ex vivo* method in cardiotoxicity research, this adds to the literature that acute exercise not only improves tumour outcomes but can also reduce the toxic side effects of chemotherapy, emphasising the value of using serological preconditioning for *in vitro* studies to investigate clinically meaningful oncology outcomes. In humans, a single acute exercise bout 24-hours before chemotherapy, decreased systemic vascular resistance and improved cardiac function in BC patients, associating with a 46% risk reduction of heart failure (Kirkham et al., 2017). Our findings add to the literature that acute exercise protection also occurs in vascular endothelial cells and this is potentially a mechanism behind the reduction in HF risk, as endothelial detriments are often initiating steps in CVD development (Verma et al., 2003).

Despite the importance of eNOS production on cardiovascular health and chemotherapy toxicity prevention, results from our study show that eNOS did not change with acute exercise in either FEC or docetaxel (despite acute exercise serum reducing apoptosis with FEC exposure). This suggests that acute exercise-induced alterations in the serum environment alone are not sufficient to induce preservation of eNOS expression with exposure to FEC-T. This will not necessarily be the case *in vivo* as exercise produces a shear

stress stimulus which is responsible for upregulation of NO production (Boo et al., 2002). Therefore, forthcoming studies should utilise an *in vitro* model of shear stress (Hattori et al., 2014) to determine whether shear stress is required to elicit acute-exercise induced changes in eNOS production and/or activation. This will determine whether acute exercise shortly before undergoing chemotherapy can elicit vascular protection via upregulated endothelial function. If this is the case, this has potential to lead to a breakthrough in oncology care as patients may be recommended to exercise prior to treatment to reduce risk of cardiovascular toxicity. This highlights the importance for research into acute exercise and endothelial function to be continued in response to these findings.

Acute exercise serum increased adhesion molecule expression when exposed to FEC and were unchanged with exposure to docetaxel, which is similar to non-chemotherapy studies whereby acute exercise upregulates ICAM-1 *in vivo* (Tinken et al., 2010), thought to be attributed to by shear stress (Chiu et al., 2004). The current findings now add to the literature that serological alterations are also likely involved in regulation of adhesion molecule expression with acute exercise. It is expected that inflammatory mediators, such as TNFα are involved in this effect as these are well-documented to stimulate adhesion molecule expression (Bernot et al., 2005) and are secreted during an exercise bout (Orange et al., 2020). Implications of preserved adhesion molecule expression for the BC patient remain unclear. However, a non-chemotherapy *ex vivo* study found that despite no change in ICAM-1 expression, immune cells have a reduced ability to adhere to the endothelium after exercise (Mills et al., 2006). Therefore, acute exercise-induced changes to immune cell function and not to adhesion molecules may be important for atherosclerosis risk reduction. The next step to expand these findings is to develop a co-culture model of leukocyte-endothelial cell interaction (as applied in the literature (Schaff et al., 2007) in the presence of acute exercise serum and chemotherapy. No other study has investigated acute exercise effects on endothelial adhesion molecule response to chemotherapy and hence, results that ICAM-1 loss with docetaxel exposure is alleviated with exercise preconditioning are novel. As this is the first study to investigate acute exercise before chemotherapy, this is a promising starting point for further investigation in a relatively new area of vascular-exercise oncology.

As with eNOS content, there was no significant between-group differences for any of the FEC-T drug conditions on endothelial wound repair. These findings are in agreement with another *in vitro* study which found no between-group difference for pre- and post-acute exercise despite transient increase in VEGF post-exercise, with VEGF having been attributed to increased HCAEC migration in the same study (Wahl et al., 2014). Therefore, this raises questions around the mechanisms of migration-proliferation across a wound and the complex interactions of other factors at play with regards to the acute exercise response. It has been found that circulating VEGF levels are significantly increased 10-minutes post-exercise (Wahl et al., 2011). Therefore, the timings of blood collection in the current study (10-minutes post-exercise) should be appropriate to elicit a response. However, increases in angiogenic factors with an exercise bout are intensity dependent (Wahl et al., 2011). Therefore, the 57% HR_{max} averaged in the current study may not be sufficient to elicit serological adaptation to produce a protective effect on endothelial cell migration-proliferation when exposed to chemotherapy. Subsequent studies should include investigating the serum for potential inhibitors of VEGF to identify possible therapeutic targets for protection against wound repair disruption to help to protect against atherosclerotic plaque development which is initiated by vascular insult without sufficient repair (Werner et al., 2008).

These original findings that acute exercise preconditioning can alleviate toxicity of FEC by inhibiting endothelial apoptosis, could shape future recommendations for exercise 'prehabilitation' to precondition the cardiovascular system before chemotherapy exposure. Our results suggest that exercise before chemotherapy is beneficial, and this may be potentially implemented into cancer care in the chemotherapy ward before treatment. This approach has been carried out previously (Kirkham et al., 2017), and therefore is deemed safe and feasible but current findings now add that this method has potential to reduce vascular toxicity with chemotherapy. Reducing vascular toxicity would in turn reduce side effects which limit chemotherapy completion and would make chemotherapy more tolerable, improving ability to withstand treatment dose, increasing treatment efficacy (Gavila et al., 2017). Reducing side effects would also improve quality of life during and after treatment and reduce acute and late health care burdens currently induced by cardiovascular toxicity. Further studies investigating the intensity, timing, and type of acute

exercise are required for optimal utilisation of 'prehabilitative' care for BC, but these studies have provided promising findings which add to the growing body of evidence for acute exercise in cardiovascular oncology.

8.5 Serological Conditioning with Serum Collected After an Exercise Training Intervention Attenuates Endothelial Cell Toxicity of FEC-T Drugs

Preconditioning endothelial cells with serum collected after an exercise intervention attenuated the apoptotic response to FEC exposure which may contribute to the 11-20% risk reduction for CVD observed in human studies of exercise interventions during and after chemotherapy for BC (Lee et al., 2019; Lewington et al., 2002; Sturgeon et al., 2014a). No known previous study of exercise interventions before or during chemotherapy have directly measured effects on endothelial damage. Therefore, our study now adds to the literature that protective effects of an exercise intervention before/during chemotherapy may be attributed to by reduced endothelial cell damage, in turn protecting against atherosclerosis, hypertension, and subsequent detriments to the whole cardiovascular system (Brower et al., 2006; Hansson, 2005; Pai & Nahata, 2000). The novel design of this *ex vivo* model allowed for these findings to be observed which otherwise may not have been possible.

Despite reduction in markers indicative of endothelial cell apoptosis, the current study found no protective effect of exercise intervention serum on eNOS bioavailability for FEC or docetaxel conditions. Exercise interventions in non-cancer populations are well-documented to improve vascular endothelial function as measured by FMD (Okada et al., 2010; Pearson & Smart, 2017; Rakobowchuk et al., 2008). Differences between findings may be due to the lack of shear stress stimuli across the endothelial monolayer in the current *ex vivo* model (Boo et al., 2002). However, in line with our study findings, Jones et al. (2013) found no significant change in vascular function from pre-to post-anthracycline chemotherapy between active or inactive groups (3 supervised cycle ergometry sessions/week at 60% to 100% of ($\dot{V}O_{2peak}$, 30 to 45 min/session, for 12 weeks). Therefore, it may well be the case that the potency of chemotherapy cannot be overcome with an exercise intervention prior to or during chemotherapy. However, an 8-week HIIT cycling intervention alongside anthracycline-chemotherapy treatment increased brachial FMD by a

clinically meaningful 4.3% in the exercise intervention group (Lee et al., 2019b). Perhaps differences in exercise protocols provide an explanation for these contrasting findings; and this creates scope for additional research into exercise protection against vascular dysfunction with chemotherapy exposure. The next step is to repeat the study on a larger scale, using different exercise programmes in BC patients to determine if there is a specific protocol required for optimal practice in BC care. This, alongside *in vivo* measurements of vascular function (FMD) will elucidate whether an exercise intervention before and/or during chemotherapy can improve vascular health and protect against cardiovascular toxicity.

There was also no difference in endothelial adhesion molecule expression with exercise intervention serum preconditioning against chemotherapy exposure. A recent review of the effects of exercise on adhesion molecule expression in humans (non-cancer) found that exercise decreased soluble adhesion molecules across a wide range of 'at risk' populations (Palmefors et al., 2014b). As with eNOS regulation, the downregulation of adhesion molecules with exercise may not be due to serological factors as the main mediator of exercise-induced regulation of adhesion molecules has also been determined to be shear stress (Tinken et al., 2010). Since *our ex vivo* model does not mimic shear stress, this cannot be determined from our findings. Therefore, this strengthens the proposal for more research utilising shear stress mimetics to determine the physiological effects of an exercise intervention prior to or during chemotherapy on adhesion molecule expression in females with BC. As no oncology research has investigated the effects of exercise on adhesion molecule expression, the current findings may well correspond to *in human* effects. However, a large randomized controlled trial in BC patients utilising serum adhesion markers is required to confirm this.

Serum preconditioning after an exercise intervention elicited protective effects on endothelial wound repair when exposed to 5-FU and docetaxel, likely due to enhanced growth factors in the serum, such as VEGF and HDL-cholesterol which promote cellular migration (Grunewald et al., 2021; Kimura et al., 2006; Palazón-Bru et al., 2021). This may be linked to the reduction in apoptosis observed with exercise intervention serum preconditioning as HDL also protects against chemotherapy toxicity in cardiomyocytes via

anti-apoptotic pathways (Durham, Chathely, Mak, et al., 2018; Durham, Chathely, & Trigatti, 2018; Durham et al., 2019; Kimura et al., 2003). Assessment of serum VEGF and HDL-cholesterol content; and the use of VEGF- and HDL-blockers in future studies will provide further evidence for the potential role of VEGF and HDL-cholesterol, respectively, in wound repair with exercise during chemotherapy. For the first time, the current findings have shown that serum preconditioning post-exercise intervention is also protective against chemotherapy-induced apoptosis in endothelial cells and this may also be via HDL and VEGF modulation (Durham, Chathely, Mak, et al., 2018; Durham, Chathely, & Trigatti, 2018; Durham et al., 2019; Kimura et al., 2003). Again, further research is required to confirm this effect. Regardless of the mechanisms, exercise protection against wound repair disruption and vascular endothelial cell death will protect against the cardiovascular disease risk observed in BC patients undergoing chemotherapy treatment (Patnaik et al., 2011a; Verma et al., 2003).

The overall toxicity of FEC-T on endothelial cells and the potential mechanisms of protection with physical activity and/or exercise is condensed in the following summary graphic (**Figure 8.6**).

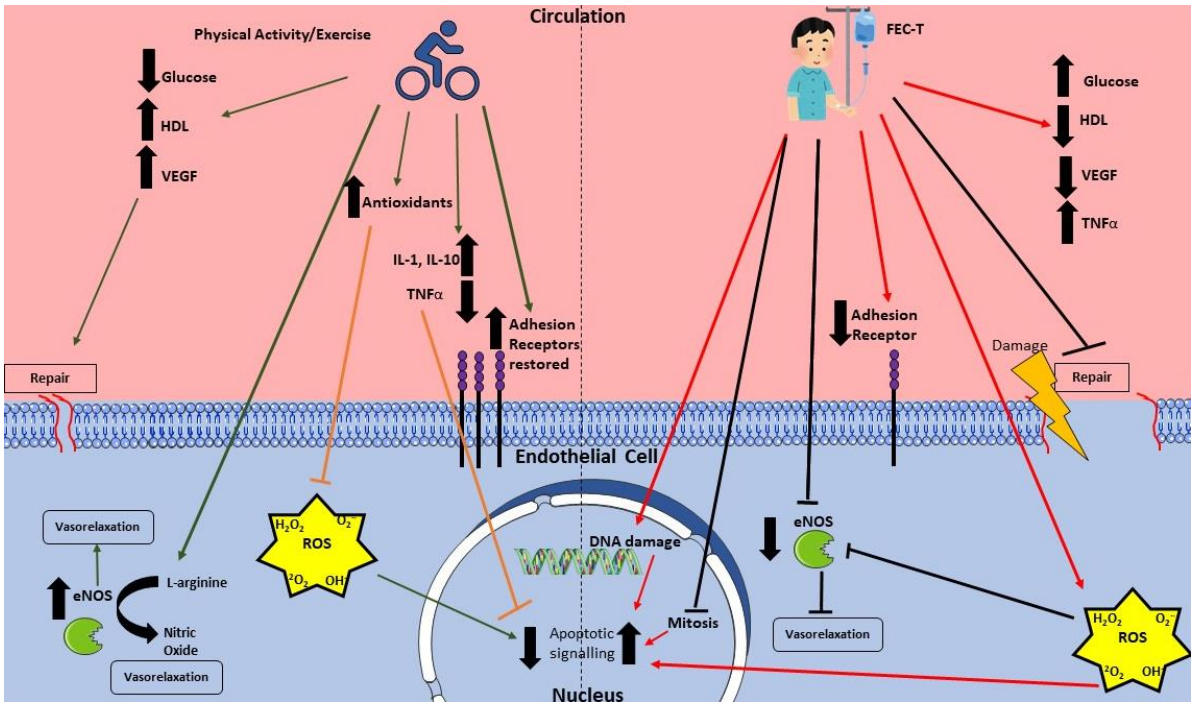

Figure 8.6. Mechanisms of chemotherapy toxicity on the endothelium and counteractive effects of exercise. *Vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), high density lipoprotein-cholesterol (HDL)*

8.6 Strengths and Limitations of the Serological Conditioning Model

8.6.1 Physiological Relevance of Chemotherapy Exposure

The serological conditioning model utilised throughout this thesis has strengths and limitations which should be noted. The main strength of this model is the endeavour to optimise physiological relevance by referring to *in-human* studies of chemotherapy exposure. Physiological concentrations of chemotherapy were used throughout experiments, in accordance with the serum concentration found in patients after exposure to each FEC-T drug (5-FU: 1.5µM (Reigner et al., 2003); epirubicin: 0.006µM (Danesi et al., 2002); cyclophosphamide: 38µM (Adams et al., 2014); and docetaxel: 6µM (Hurria et al., 2006)). Similarly, time-points for cell washes were in accordance with the time-points whereby the drugs were diminished in bloodstream of humans (5-FU: 3- hours (Reigner et al., 2003); epirubicin: 1- hour (Danesi et al., 2002); cyclophosphamide: 12-hours (Adams et al., 2014); docetaxel: 24-hours (Hurria et al., 2006); FEC: 4-hours). Lastly, analysis time-points were also relevant to the literature findings of optimal chemotherapy potency 5-FU: 3 and 12 hours; epirubicin: 4 and 12 hours; cyclophosphamide: 3 and 6 hours (Chow & Loo, 2003); and docetaxel: 24 and 48 hours (Morse et al., 2005); FEC: 4 and 12 hours, based on our own findings). Taken together, these *in-human* findings have shaped the development of the serological conditioning model utilised throughout this thesis, maintaining physiological relevance throughout. Furthermore, the initial study carried out in HUVECs was progressed to a more physiologically relevant cell model – HCAECs – which are derived from the arterial human vasculature, making findings more applicable to *in-human* studies of chemotherapy-induced CV toxicity. The coronary artery is particularly susceptible to damage and dysfunction (Winther et al., 2016) which can lead to development of atherosclerosis, myocardial ischaemia and myocardial infarctions (Hansson, 2005; Thygesen et al., 2012), as these are the vessels supplying blood to the heart itself. Hence, investigation of HCAECs maintained the physiological relevance of the serological conditioning model.

8.6.2 Physiological Relevance of Endothelial Cells

Furthermore, HUVECs or HCAECs do not originate from cancer patients. This said, it appears that endothelial function is similar in cancer populations before treatment as in non-cancer populations, with Duquaine et al. (2003) finding that a single dose of anthracycline chemotherapy reduced FMD in BC patients from 6.5% (typically seen in healthy populations (Ghiadoni et al., 2012)) to 2.5% (typically seen in CVD populations (Benjamin et al., 2004)). This is echoed in a study whereby prior to chemotherapy, absolute forearm blood flow in the BC patients did not differ from non-cancer controls (Fredslund, Buus, Skjold, et al., 2021). Therefore, the difference in endothelial cell health between cancer and non-cancer patients is not the cancer itself but the treatment process, and hence, endothelial cells do not need to be derived from cancer patients to be relevant to the question of chemotherapy exposure effects.

8.6.3 Comparison of the Current Serological Model with Other Models

There is now a wealth of studies utilising serum conditioning models. Other studies preconditioned cells for 24-hours with 5-20% pooled serum in their cell culture media (Allen et al., 2021; Orange et al., 2020; Wahl et al., 2014) and hence, the reason for selecting 24-hours and 5% pooled serum for the current studies. Serological models have been used in exercise-oncology research and have been used to determine whether changes in systemic environment with acute exercise contribute to the reduced cancer risk with acute exercise. The general conclusion is that acute exercise serum reduces cancer cell growth (Orange et al., 2020). Utilisation of these serological models provided potential mechanisms for the *in-human* observations of reduced cancer risk which may not have been possible without this model as several serum markers can be analysed without repeated invasive testing in humans (Orange et al., 2020). The field of exercise science has also utilised this method for non-cancer studies of high intensity exercise and endothelial cell health, allowing determination of the mechanisms of exercise-induced cardiovascular health benefits (Wahl et al., 2014). *Ex vivo* serum preconditioning has not only been used for exercise and oncology research but has also recently been used in wider disciplines, including the field of aging (Allen et al., 2021). This model has proved useful in pinpointing mediating mechanisms of aging at a cellular level, uncovering potential therapeutic targets to protect

against age-related disease (Allen et al., 2021). As a whole, serological conditioning *ex vivo* models are widely accepted to provide mechanistic links before moving to *in human* studies or to further explain observations from *in human* studies. The studies performed in this thesis are the first to investigate exercise sera before chemotherapy exposure, and therefore are a starting point for further investigation in a relatively new area of vascular-exercise oncology. Additionally, the model developed here could be used in future investigation of chemotherapy effects on other cell types, including cardiomyocytes, to determine protective effects of serological preconditioning.

8.6.4 Relevance of Participants to Oncology Studies

Throughout all 3 studies, human participants were non-cancer females and therefore, serum was not derived from cancer survivors. As it is chemotherapy and not the disease itself that causes endothelial dysfunction (Duquaine et al., 2003b; Fredslund, Buus, Højgaard Skjold, et al., 2021), non-cancer sera was deemed suitable for analysis. The 'multi-hit' hypothesis suggests that it is not only the chemotherapy itself but a number of factors which contribute to an elevated CV risk within BC survivors, including pre-existing traditional CVD risk factors (Kirkham et al., 2019). Hence, it is of interest to investigate protective effects of exercise in individuals who are at risk of developing CVD. The participants in the exercise intervention study are considered 'at risk' of CVD due to presence of hyperglycaemia, hypertension, obesity, and hyperlipidaemia (Dahlöf, 2010). These participants are relevant to BC patients as their characteristics are similar, with 63–65% BC patients having overweight/obesity, 30–33% hypertension, 4% diabetes, and 11% hypercholesterolemia (Park et al., 2017). Hence, this allows conclusions to be drawn that for those who are already at risk of developing cardiovascular toxicity when exposed to chemotherapy (Kirkham et al., 2019), an exercise intervention may provide some protective benefits for the vascular endothelium.

8.6.5 Serological Adaptations to Physical Activity/Exercise Does Not Provide the Whole Picture

A potential problem for the current study is that exercise-induced protection of endothelial function *in vivo* may not be due to systemic serum adaptations to exercise. Differences in

serum composition between active and inactive/pre- and post- samples have not been investigated in this study. If there is no real difference in composition, then this may be why there is a lack of exercise-induced protection against eNOS bioavailability and activation. The increased eNOS and phospho-eNOS expression seen in other studies may not be due to systemic adaptations and perhaps require exercise-induced increases in shear stress to induce adaptations in endothelial function and health (Gielen et al., 2011). During exercise, blood flow is increased, and this may be responsible for increases in eNOS production with exercise training (Balligand et al., 2009). As shear stress has not been mimicked in this cell culture model, it is not possible to provide a conclusion on this. Therefore, future studies should utilise a shear stress mimetic as applied elsewhere in the literature (Hattori et al., 2014).

8.7 General Limitations

8.7.1 Use of 2D Cell Culture Models

Despite the highest efforts to create physiological relevance, the main limitation to this study is that our cell culture model may not fully reflect *in vivo* conditions. This is always a limitation for *in vitro* experiments. A limitation of all studies is the use of 2D cell culture models, which do not fully mimic the *in vivo* endothelial microenvironment. Three-dimensional cell cultures better recapitulate *in vivo* endothelial physiology, and the physical characterizes of a blood vessel (Aizawa, Wylie, & Shoichet, 2010). 3D cell culture models (Aizawa et al., 2010) or vessels in organ baths (Tarhan et al., 2007) would better reflect *in vivo* conditions and provide stronger evidence on the effect of physical activity/exercise on the response of the endothelium to chemotherapy for BC.

8.7.2 Flow Cytometric Determination of Endothelial Health and Function

With regards to the methods of analysis, the flow cytometry antibodies used were unable to be used in one panel due to the same fluorophore being used, and hence, two separate panels were created in each of the studies. This means that adhesion molecule data was gate on CD31 only and could not be gated on Annexin V or cleaved caspase-3 and hence, the analysis includes both live and dead cells. Analysis of co-expression of adhesion molecules,

eNOS, and apoptotic markers is required to fully explain the changes observed for adhesion molecule expression.

Furthermore, due to the unexpected nature of findings for adhesion molecule expression changes, it would have been advantageous to confirm and expand the observed changes in adhesion molecules in with another technique, such as PCR for intracellular transcription analysis, and Western Blotting (Chen, Esselman, Jump, & Busik, 2005) or fluorescence microscopy (Carman, Jun, Salas, & Springer, 2003) for intracellular and cell surface protein expression. Investigation using these techniques is required to elucidate the full picture of ICAM-1 role in chemotherapy-induce endothelial toxicity and exercise protection against toxicity.

8.7.3 Analysis of Wound Repair by Scratch Assay

Although the mechanical technique used in the protocol for the wound healing assay is simple, cost-effective, and has a relatively high through-put, there are some limitations to this method. With no automation, reproducibility poses as a problem, with minimal control over wound geometry, boundaries, or surface properties (Riahi, Yang, Zhang, & Wong, 2012). As each user may apply differing pressures when inflicting a wound across the monolayer, there is potential for high inter-individual variability and hence, differences would occur between laboratories.

Another limitation of analysing endothelial wound repair via the scratch assay method is that it cannot be determined whether the repair is due to cellular migration or proliferation. Use of time-points under 24-hours reduces the contribution of proliferation (Martinotti & Ranzato, 2020). However, to truly reduce the risk of contribution of cell proliferation, Mitomycin C can be used to inhibit DNA replication (Martinotti et al., 2017). However, this method was not available for these studies and data must therefore be interpreted as both migration and proliferation. Furthermore, the endothelial wound healing assay has the limitation of only assessing the ability of resident endothelial cells to repair. The potential effects of chemotherapy exposure on other relevant cell types, such as endothelial progenitor cells or angiogenic T cells have not been assessed, despite their importance for endothelial repair *in vivo* (Kwee, Budina, Najibi, & Mooney, 2018; Suh et al., 2005).

8.7.4 Sample Size

The sample size of studies in *Chapter 5* is low (n=3). This was the preliminary study for initial evidence for benefits of physical activity/exercise by which the subsequent intervention study (with a larger sample size) would be based. A sample size of 8 has been deemed acceptable in other serological conditioning models (Caldeira-Dias et al., 2018, 2019; Sandrim et al., 2016). However, it was not possible to recruit a further 5 participants due to COVID-19 restrictions halting recruitment. Repeating experiments with larger sample sizes would reduce inter-individual variability within pooled samples to ensure physical activity levels are the only contributing factor to the results. However, since similar beneficial results of serum conditioning were observed within the exercise intervention study (n=15), results were considered suitable for presentation within the PhD thesis.

Additionally, the sample size of cell culture replicates (n=3) is another limitation to these studies which must be noted. Three biological replicates are considered the minimal required for sound inferential statistical analysis which is important for addressing how widely the results can be generalized (Blainey, Krzywinski, & Altman, 2014; Naegle, Gough, & Yaffe, 2015). Although the datasets throughout these studies can be interpreted with confidence, increasing the number of replicates would increase statistical power and increase confidence further (Blainey et al., 2014; Naegle et al., 2015). However, 3 was the chosen number of replicates as studies had to adhere to a finite budget and therefore, costs of cell culture and flow cytometry reagents had to be balanced with the quality of the research output.

8.8 Future Research

Data presented in *Chapter 4* have shown that FEC-T chemotherapy drugs negatively impact endothelial cells and that serum from habitually active females helps to alleviate some of the observed toxicity.

In respect to these observations, future studies should address the following research questions:

1. Since endothelial damage occurs with FEC-T exposure and endothelial damage and dysfunction is pertinent to cardiovascular disease development, should endothelial function be utilised as a measurement of early cardiovascular toxicity in humans?
2. What are the consequences of FEC-T chemotherapy exposure on other cells of the cardiovascular system, including cardiomyocytes?

Data observed in *Chapter 5* have shown that serum from physically active females can alleviate some of the endothelial cell toxicity induced by exposure to FEC-T drugs *in vitro*.

In respect to these observations, future studies should address the following research questions:

1. Are findings of physical activity protection applicable *in vivo*? What are the *in-human* vascular consequences of habitual physical activity before and during chemotherapy for BC? How much and what type of physical activity is required before a vascular benefit is observed? Can current physical activity levels be offset with an intervention?
2. What are the consequences and mechanisms of upregulation of adhesion molecules with increased physical activity levels?
3. Are findings applicable to other chemotherapy treatment regimens?

From *Chapter 6*, it has been observed that serum collected after a single acute exercise session can help to mitigate effects of chemotherapy by alleviating apoptosis of endothelial cells and effects on ICAM-1 but had no effect on endothelial function, including eNOS production or wound repair.

In respect to these observations, future studies should address the following research questions:

1. What are the upstream effects of protection of endothelial cells from apoptosis? What component of the serum is protecting against apoptosis?
2. What are the consequences of upregulated ICAM-1 expression? Do immune cells adhere more readily to adhesion molecules or does acute exercise reduce immune cell ability to adhere to the endothelium?
3. Are shear stress mimetics required for an effect on endothelial function and wound repair? Or is there truly no effect of acute exercise on these parameters?

The main findings from *Chapter 7* are that an exercise intervention in 'at risk' women helped to alleviate FEC-T induced endothelial toxicity, as shown by reduced apoptosis, alongside preserved wound repair with exposure to docetaxel.

In respect to these observations, future studies should address the following research questions:

1. Are the reductions in apoptosis and the preservation of wound repair mechanistically linked? Do these effects occur *in human*?
2. What components of the serum are causing the protective effects of exercise training? Could these be utilised as future therapeutic targets to reduce toxicity burden?
3. Are results similar in cancer patients as there could be differing responses to exercise between cancer and non-cancer populations?

8.9 Conclusion

The overall findings from this PhD thesis are that FEC-T drugs cause endothelial cell toxicity *in vitro* and that habitual physical activity, acute exercise, and an exercise intervention can help to partially alleviate this toxicity. Increased physical activity/exercise reduces endothelial cell apoptosis. However, only habitual physical activity observed benefits on endothelial cell eNOS content. Moreover, endothelial wound repair is partially preserved by both habitual physical activity and an exercise intervention, dependent upon the drug of exposure, with docetaxel receiving the most benefit. The repercussions of the upregulation of adhesion molecules with habitual physical activity and acute exercise preconditioning are less clear and require further investigation. However, overall, endothelial cell health is

slightly preserved with physical activity/exercise serum preconditioning before FEC-T exposure, adding to the literature that being active before/during treatment for BC is beneficial in protecting against cardiovascular toxicity.

As only 27% of women with BC are physically active compared with 44% of non-cancer counterparts (Clark et al., 2017), cardiotoxicity remains a concern for BC populations. To tackle this problem, exercise interventions during chemotherapy for BC should be implemented, with advice to clinicians and patients required to be specific to the particular chemotherapy regimen.

Chapter 9

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Appendix

Healthy Female Participants Needed!

Do you want to be involved in cancer research?

We are looking for healthy exercise trained and untrained volunteers

You will be required to visit our lab to provide a blood sample and perform a quick fitness test.

You must be:

- **Female**
- **Healthy**
- **Caucasian**
- **Aged 18-35 years**
- **BMI: 18kg/m^2 to 25kg/m^2**
- **Taking contraceptive pill**

For further information contact:

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0131 455 2487

Appendix 2.

Consent Form:

Chronic Exercise protection against cardiovascular toxicity of chemotherapy

Edinburgh Napier University requires that all persons who participate in research studies give their written consent to do so. Please read the following and sign it if you agree with what it says.

1. I freely and voluntarily consent to be a participant in the research project on the topic of exercise to attenuate cardiovascular toxicity of chemotherapy to be conducted by Marie Mclaughlin who is a PhD student at Edinburgh Napier University.

2. The broad goal of this research study is to determine if there are protective effects of exercise on cardiac and vascular cells during chemotherapy treatment. Specifically, I have been asked to complete a physical activity questionnaire which will determine if I am exercise 'trained' or 'untrained'. I will then be required to provide a blood sample (which will be used for research purposes and no DNA analysis shall be performed). Immediately afterwards, I will perform a VO2max test. This will mark the completion of my involvement in the study.

3. I have been told that my responses will be anonymised. My name will not be linked with the research materials, and I will not be identified or identifiable in any report subsequently produced by the researcher.

4. I am aware that the research student and their supervisors will have access to my personal data throughout the study.

5. I also understand that if at any time during the project I feel unable or unwilling to continue, I am free to leave. That is, my participation in this study is completely voluntary, and I may withdraw from it without negative consequences. However, after data has been anonymised or after publication of results it will not be possible for my data to be removed as it would be untraceable at this point.

6. In addition, should I not wish to answer any particular questions, I am free to decline.

7. I have been given the opportunity to ask questions regarding the study and my questions have been answered to my satisfaction.

8. If I become incapacitated during this study, I will be withdrawn, and no more research data shall be obtained for this study. All data already collected shall be retained and used.

9. I have read and understand the above and consent to participate in this study. My signature is not a waiver of any legal rights. Furthermore, I understand that I will be able to keep a copy of the informed consent form for my records.

Participant's Signature Date

I have explained and defined in detail the research procedure in which the respondent has consented to participate. Furthermore, I will retain one copy of the informed consent form for my records.

Researcher's Signature Date

Appendix 3.

Edinburgh Napier UNIVERSITY

Consent Form: Effects of Acute Exercise on Vascular Cells

Edinburgh Napier University requires that all persons who participate in research studies give their written consent to do so. Please read the following and sign it if you agree with what it says.

1. I freely and voluntarily consent to be a participant in the research project on the topic of exercise to attenuate cardiovascular toxicity of chemotherapy to be conducted by Marie Mclaughlin who is a PhD student at Edinburgh Napier University.
2. The broad goal of this research study is to determine if there are protective effects of exercise on vascular cells during chemotherapy treatment. Specifically, I have been asked to perform a single low-intensity exercise session, by following a MacMillan exercise DVD. This will last around one hour. Before and afterwards, I will be required to give a blood sample, which will be used for research purposes and no DNA analysis shall be performed. This will mark the end of my participation in this study.
3. I have been told that my responses will be anonymised. My name will not be linked with the research materials, and I will not be identified or identifiable in any report subsequently produced by the researcher.
4. I am aware that the research student, and their supervisors will have access to my personal data throughout the study.
5. I also understand that if at any time during the project I feel unable or unwilling to continue, I am free to leave. That is, my participation in this study is completely voluntary, and I may withdraw from it without negative consequences. However, after data has been anonymised or after publication of results it will not be possible for my data to be removed as it would be untraceable at this point.
6. In addition, should I not wish to answer any particular questions, I am free to decline.
7. I have been given the opportunity to ask questions regarding the study and my questions have been answered to my satisfaction.
8. If I become incapacitated during this study, I will be withdrawn, and no more research data shall be obtained for this study. All data already collected shall be retained and used.
9. I have read and understand the above and consent to participate in this study. My signature is not a waiver of any legal rights. Furthermore, I understand that I will be able to keep a copy of the informed consent form for my records.

Participant's Signature Date

I have explained and defined in detail the research procedure in which the respondent has consented to participate. Furthermore, I will retain one copy of the informed consent form for my records.

Researcher's Signature Date

Appendix 4.

AHA/ACSM Health/Fitness Facility Pre-participation Screening Questionnaire

Assess your health status by marking all true statements

History

You have had:

a heart attack

heart surgery

cardiac catheterization coronary angioplasty (PTCA)

Pacemaker/implantable cardiac defibrillator

rhythm disturbance

heart valve disease

heart failure

heart transplantation

congenital heart disease

Symptoms:

You experience chest discomfort with exertion. You experience unreasonable breathlessness

You experience dizziness, fainting, or blackouts You take heart medications

Other health issues

You have diabetes

You have asthma or other lung disease

You have burning or cramping sensation in your lower legs when walking short distances You have musculoskeletal problems that limit your physical activity. You have concerns about the safety of exercise

You take prescription medication(s).

You are pregnant.

If you marked any of these statements in this section, consult your physician or other appropriate health tare provider before engaging in exercise. You may reed to use a facility with a medically qualified staff.

Cardiovascular risk factors

You are a man older than 45 years.

You are a woman older than 55 years, have had a hysterectomy, or are postmenopausal

You smoke, or quit smoking within the previous 6 months.

Your blood pressure is >140190 mm Hg.

You do not know your blood pressure.

You take blood pressure medication.

Your blood cholesterol level is >200 mg/dl.

You do not know your cholesterol level.

You have a close blood relative who had a heart attack or heart surgery before age 55 (father or brother) or age 65 (mother or sister).

You are physically inactive (i.e., you get <30 minutes of physical activity on at least 3 days per week).

You are >20 pounds overweight

None of the above

If you marked two or more of the statements in this section you should consult you physician or other appropriate health rare provider before engaging in exercise. You might benefit from using a facility with a professionally qualified exercise staff to guide your exercise Program.

You should be able to exercise safely without consulting your physician or other appropriate health care provider in a self-guided program or almost any facility that meets your exercise program needs.

Modified from American College of Sports Medicine and American Heart Association. ACSM/AHA Joint Position Statement: Recommendations for cardiovascular screening, staffing, and emergency policies at health/fitness facilities. Medicine and Science in Sports and Exercise 1998:1018.

Appendix 5.

Physical Activity Readiness Questionnaire (PAR Q) Long version

When using this form, you need to state:

Why you are collecting this information.
What you are going to do with this information (how you will store this).
Your policy for destroying this information (within a period of time or once the client has left).

Your Personal Details

Client Name: _____ DoB: _____

Address: _____

_____ Postcode: _____

Email: _____ Phone: _____

Emergency Contact Details

Name:

_____

Address: _____

_____ Postcode: _____

Email: _____ Phone: _____

Your Health Goals

1. What health goals would you like to achieve in the next 3 months?

_____

2. Name 3 things you could do in order to improve your health?

_____

What are your main reasons for starting a fitness programme?

General conditioning ☐ Muscular strength ☐ No time ☐

Weight /fat loss ☐ Aerobic fitness ☐ Appearance ☐

Stress management ☐ FlexibilityImprove self-esteem ☐ ☐

Other ☐

How would you describe your general health and fitness?

_____

Have you ever done any structured exercise? Yes / No

If '**Yes' what did you do?**

_____

What type of exercise do you enjoy the most?
What type of exercise do you dislike the most?

_____

_____

What would you say are the main barriers preventing you from exercising?

Lack of facilities ☐ No motivation ☐ No time ☐

Injury/illness ☐ Unfit ☐ Appearance ☐

Lack of knowledge ☐ Family ☐ Work ☐

Diet and Nutrition

On a scale of 1-10 **(with 1 being poor and 10 being excellent)** how would you assess the quality of your eating habits?

Would you like any help or advice in changing the quality of your eating habits? **Yes / No**

Do you follow any particular diet or eating patterns?

Lifestyle

Do you drink alcohol? **Yes / No**

Do you smoke? **Yes / No**

If you answered 'Yes', would you like help or advice to change these habits? **Yes / No**

Medical History

Have you had a major illness or injury in the last 5 years **Yes / No**

If 'Yes' please give details

_____

_____

Are you receiving treatment for any diagnosed medical condition? **Yes / No**

If 'Yes' please give details

_____

_____

Are you taking any prescription medication? **Yes / No**

If 'Yes' please give details

_____

_____

Please indicate if you ever experience any of the following symptoms. Do you:

Ever get unusually short of breath with very light exertion? □ ☐

Ever have pain, pressure, heaviness or tightness in the chest area? □ ☐

Regularly have unexplained pain in the abdomen, shoulders or arm? ☐

Please indicate if you ever experience any of the following symptoms. Do you:

Ever have severe dizzy spells or episodes of fainting? □ ☐

Regularly get lower leg pain during walking that is relieved by rest? □ ☐

Ever experience palpitations or irregular heartbeats? □ ☐

Are you currently pregnant or have you given birth in the last 6 months? **Yes / No**

Structural Health

Please indicate on the figures below any aches, pains or problem areas.

Please give details of any areas indicated

_____

_____

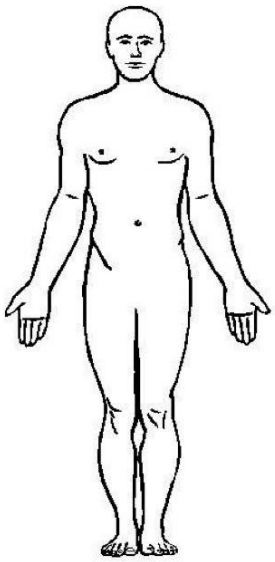
_____

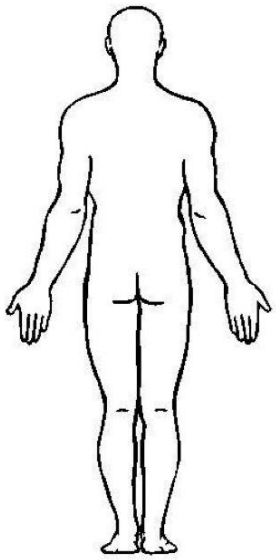
_____

Are any of these injuries aggravated by exercise? **Yes / No**

Are you currently receiving treatment for any structural problem? **Yes / No**

Please indicate any other health problems you suffer from which you have not already mentioned.





I can confirm that I have answered all questions honestly and that the information given is correct.

Signature: _____ Print name: _____ Date: _____

Note: This PAR Q becomes invalid should your condition change.

93327

Appendix 6

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others

by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective.* Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

Yes ☐

☐ No → *Skip to PART 2: TRANSPORTATION*

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ days per week

☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

_____ hours per day _____ minutes per day

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

_____ days per week

☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ hours per day _____ minutes per day

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ days per week

☐ No job-related walking → Skip to PART 2: TRANSPORTATION

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ hours per day _____ minutes per day

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ days per week

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ hours per day _____ minutes per day

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ days per week

☐ No bicycling from place to place → ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ hours per day _____ minutes per day

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ days per week

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK,***

HOUSE MAINTENANCE, AND CARING FOR FAMILY

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ hours per day _____ minutes per day

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ days per week

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ hours per day _____ minutes per day

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ days per week

☐ No moderate activity in garden or yard → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ hours per day _____ minutes per day

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ days per week

☐ No moderate activity inside home → ***Skip to PART 4: RECREATION,***

SPORT AND LEISURE-TIME PHYSICAL ACTIVITY

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ hours per day _____ minutes per day

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ days per week

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ hours per day _____ minutes per day

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ days per week

☐ No vigorous activity in leisure time → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ hours per day _____ minutes per day

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ days per week

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?

_____ hours per day _____ minutes per day

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ hours per day _____ minutes per day

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ hours per day _____ minutes per day

This is the end of the questionnaire, thank you for participating.

.

Appendix 7. Phospho-eNOS Validation Experiment

Since phosphorylation of eNOS Serine protein 1177 is essential for production of NO, investigation of eNOS phosphorylation state is important to determine endothelial NO production. Phosphorylation state of proteins are usually assessed using Western Blot technique. Western Blotting has the drawback that it is a time-consuming process by which proteins are analysed one at a time. Flow cytometry is a faster and more precise method of assessment as this allows analysis of multi-parameters and sub-populations within cells and therefore, is a more advantageous technique compared with Western Blotting. Despite its ease of application and an available antibody for phospho-eNOS, a standardized method of flow cytometric assessment has not yet been developed. Therefore, a validation experiment was carried out. Ca^{2+} ionophore (A23187, Sigma-Aldrich, Gillingham, UK, product code: C7522) was used to upregulate phosphorylation of eNOS (Motley et al., 2007).

10.7.1 Methodology

HUVECs (Thermofisher Scientific, Waltham, USA, product code: C0035C) were cultured in ECGM (Sigma-Aldrich, Gillingham, UK, product code: 211-500) in T-75 flasks as previously described. At 80% confluence, passage 5 HUVECs were detached from T-75s using trypsin/EDTA (Gibco, Thermofisher Scientific, Waltham, USA, product code: 15400054) solution and centrifuged to create a cell pellet, as previously described. The supernatant was discarded, and cells resuspended in 1ml PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002). A cell count was performed to determine volume required for 50,000 HUVEC cells, which were transferred to Eppendorf tubes. Cells were resuspended in 1ml PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002) containing 5µM A23187 Ca^{2+} ionophore (Sigma-Aldrich, Gillingham, UK, product code: C7522) and incubated for 60min at 4^{o}C in the dark (Feron, Saldana, Michel, & Michel, 1998). Cells were washed twice with 1ml PBS by centrifugation for 5 minutes at 400 x *g* at21^{o}C. The supernatant was carefully discarded, and the cell pellet was resuspended in 100µl PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002) for preparation by flow cytometry analysis. Control cells did not receive calcium ionophore and were immediately washed and resuspended in 100µl PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002) for flow cytometry preparation.

Samples were prepared for flow cytometry analysis by antibody staining. HUVECs were incubated with 10µl anti-CD31-FITC (BioLegend, USA, product code: 303104) mAb solution for 30 minutes at 4^{o}C in the dark. After incubation, cells were washed twice with 100µl PBS (Invitrogen, Thermofisher, USA, product code: 003002) and centrifuged for 5 minutes at 400 x g at 21^{o}C. Supernatant was carefully discarded, and cells resuspended in 250µl of BD fixation/permeabilization solution (BD Biosciences, USA, product code: 554714) and incubated for 20 minutes at 4^{o}C in the dark. Two cell washes were then performed, using 100µl of permeabilization wash buffer (BD Biosciences, USA, product code: 554714) and centrifuging for 5 minutes at 400 x g, 20^{o}C. The supernatant was carefully discarded, and cells resuspended in 100µl permeabilization wash buffer (BD Biosciences, USA, product code: 554714). 10µl of eNOS-PE (Miltenyi Biotec, Bergisch Gladbach, Germany, product code: 130-106-787) and anti-phospho-eNOS-PE (Biorbyt, UK, product code: orb124607) mAb solutions were added to their corresponding FACS tubes and incubated for 30 minutes at 4^{o}C in the dark. Two cell washes were then performed using permeabilization wash buffer (BD Biosciences, USA, product code: 554714) as before, and cells resuspended in 500µl PBS (Invitrogen, Thermofisher, USA, product code: 003002) for flow cytometric analysis. Flow cytometer setup and calibration were performed using CS&T beads (BD Biosciences, USA). For sample acquisition, the cells were set as stopping gate of 10000 events. Data were acquired using FACSDiva 6.0 Software (BD Bioscience, USA). Fluorescence compensation was performed using BD CompBeads Set Anti-Mouse Ig.

Data were analysed using FACSDiva 6.0 Software (BD Bioscience, USA) to create dot plots and histograms which were gated on population, singlets, and CD31, to generate the numerical data for phospho-eNOS expression. Experiments were performed in biological triplicate and pooled, allowing statistical analysis to be performed. Shapiro-Wilks test of normality were performed using Graph Pad Prism (Version 8.0). All data was determined to be normally distributed. Two-way ANOVA was performed to determine the effect of Ca^{2+} ionophore exposure on eNOS and phospho-eNOS expression. Statistical significance was assumed if $p < 0.05$.

10.7.2 Results

Two-way ANOVA revealed that there was a statistically significant increase in phospho-eNOS expression with A23187 exposure (2439 ± 191.4 to 4192 ± 463.7 AU; p<0.01). There was no significant change in eNOS expression with A23187 exposure, (2166 ± 223.0 to 2922 ± 308.5; p=0.23).

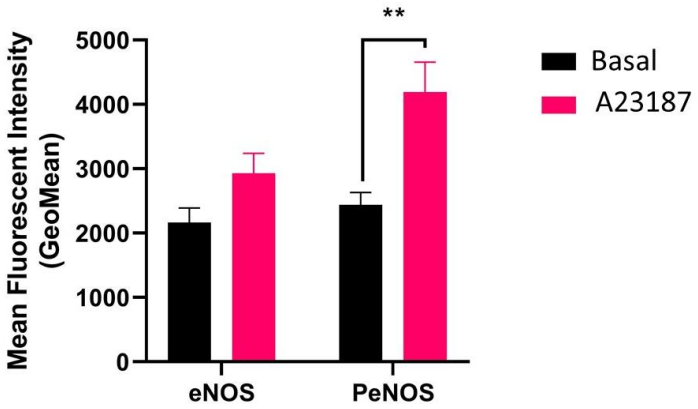


Figure 12. eNOS and phospho-eNOS expression with and without presence of A23187 Ca^{2+} ionophore. *Data are expressed as mean ± standard error of mean (SEM). *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001.*

Our findings indicate that the anti-phospho-eNOS-PE (product code: orb124607; Biorbyt, UK) does indeed detect phosphorylation state of eNOS, as desired. When measured by Western Blot, A23187 Ca^{2+} ionophore upregulates phosphorylation of eNOS Serine1177 protein (Motley et al., 2007). The current findings validate that this is also the case when A23187 Ca^{2+} ionophore-induced upregulation of phosphorylation of eNOS Serine1177 protein is analysed using flow cytometry. The current data also shows a non-significant increase in eNOS expression with A23187 Ca^{2+} ionophore exposure (**Figure 10**). This is consistent with Western Blot analysis, which shows no change in eNOS expression with A23187 Ca^{2+} ionophore exposure (Motley et al., 2007). Therefore, due to the detection of an increase in phospho-eNOS (**Figure 10**), the current findings validate the mAb for use in flow cytometric analysis of eNOS Serine 1177 protein phosphorylation state.

Compared to Western Blot technique, flow cytometry is more precise and time-efficient, with less complicated and faster sample preparation, and a rapid sample acquisition speed. Therefore, this is a preferred method over Western Blot and hence, anti-phospho-eNOS-PE

(product code: orb124607; Biorbyt, UK) was added to the panel of monoclonal antibodies used to detect differences in endothelial cell health throughout the experiments outlined in this thesis.

Appendix 8. Endothelial Cell Adhesion Molecule Analysis by Flow Cytometry

The endothelial adhesion molecule, ICAM-1 was identified as being integrally important for atherosclerosis risk as this molecule plays a key role in the binding of leukocytes to the endothelium (Mudau et al., 2012a). Chemotherapy has been shown to increase ICAM-1 levels (Meijer, 2009; Mills et al., 2004), likely contributing to the elevated CVD risk in BC patients (Patnaik et al., 2011a). Hence, ICAM-1 is a marker of interest for assessing the effects of chemotherapy on vascular endothelial cell health, and can be measured using flow cytometry (Gräbner, Till, & Heller, 2000).

For investigation of cellular surface adhesion molecules, it was recognised as a potential problem that trypsinisation partially hydrolyses cell surface ICAM-1 protein expression (Gräbner et al., 2000). This problem was overcome by fixing cells with formaldehyde and staining the endothelial monolayer before cell detachment (Gräbner et al., 2000). However, a less harsh detachment reagent could be used in future. Accutase is a less harsh reagent for cell detachment (Nowak-Terpiłowska, Śledziński, & Zeyland, 2021) and therefore, an experiment was set up to determine the difference between in-plate staining and out-of-plate staining with accutase detachment on the detection of endothelial cell adhesion molecule expression, using TNF-α to activate the endothelial cells and upregulate the surface expression of ICAM-1.

10.8.1 Methodology

HCAECs (50,000 cells) (Sigma-Aldrich, UK, product code: 300-05A) were plated in a 24-well plate at passage 8 with 1ml MesoEndothelial Growth Medium (MEGM; Sigma-Aldrich, Gillingham, UK, product code: 212-500) and incubated for 24-hours at 37^{o}C, 5% CO_2, to ensure full cell attachment. For upregulation of ICAM-1, the monolayers were stimulated for 12 hours with 4ng·ml^{-1} TNF-α (Thermofisher Scientific, Waltham, USA, product code: A42549) (Y. H. Chen et al., 2001). After 24 hours, 0.4µl of TNF-α (Thermofisher Scientific, Waltham, USA, product code: A42549) and 499.6µl fresh MEGM (Sigma-Aldrich, Gillingham, UK, product code: 212-500) were added to each well (controls receive 1000µl MEGM only). At 6-hour time-point, media was removed, and cells washed with 200µl PBS. For out-of-plate staining, cells were detached using 200µl trypsin/EDTA (Gibco, Thermofisher Scientific, Waltham, USA, product code: 15400054) or 200µl accutase (Gibco, StemPro, Thermofisher

Scientific, Waltham, USA, product code: A111050), for 1-minute, according to their respective wells. 100µl media was then added to wells to neutralise the trypsin. 300µl from each well was transferred into 96-well v-bottom plate, centrifuged for 3 minutes at 300 x *g*, 21^{o}C. The supernatant was discarded, and cell pellets resuspended in 100µl PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002) for flow cytometry analysis. For in-plate staining, HCAEC monolayers were washed with 200µl PBS and incubated with 100µl 0.5% formaldehyde (made from paraformaldehyde) for 5 minutes at 4^{o}C for fixation, according to the literature (Gräbner et al., 2000). Monolayers were then washed with 200µl PBS and re-incubated with 100µl 0.1% BSA (bovine serum albumin) PBS containing flow cytometry antibodies. For both out-of-plate and in-plate staining, HCAECs were incubated with 1µl of anti-CD54- Alexa Fluor 488 mAb (anti-ICAM-1; BioLegend, USA, product code: 322713) solution for 30 minutes at 4^{o}C in the dark.

For out-of-plate staining, HCAECs were washed with 100µl PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002) and centrifuged for 3 minutes at 300 x *g*, 4^{o}C. For in-plate staining, the cells monolayer was washed with 200µl PBS and detached using accutase, as above, before centrifugation at 300 x *g* for 3 minutes at 4^{o}C. The supernatant was discarded, and cell pellet resuspend in 100µl PBS (Invitrogen, Thermofisher, Waltham, USA, product code: 003002). The 96-well v-bottom plate was analysed using High Throughput System (HTS) on a 12-colour flow cytometer (FACS Celesta, BD Biosciences, USA). For sample acquisition, the cells were set as stopping gate of 10000 events on the High-Throughput System (HTS). HTS was set to consistently standardized parameters: flow rate µl/sec = 2.0; sample volume = 100 µl; mix volume = 100 µl; mix speed = 75 µl; number of mixes = 5; wash volume = 300 µl. Flow cytometer setup and calibration were performed using CS&T beads (BD Biosciences, USA). For sample acquisition, the cells were set as stopping gate of 10000 events. Data were acquired using FACSDiva 6.0 Software (BD Bioscience, USA). Fluorescence compensation was performed using BD CompBeads Set Anti-Mouse Ig (details provided in *Chapter 3.2.2.7*).

10.8.2 Results

The ANOVA analysis revealed that TNF-α significantly increased expression of ICAM-1 (p<0.001). ICAM-1 expression increased with TNF-α stimulation to a greater extent with detachment using accutase compared to trypsin (**Figure 11**) (accutase: 51525 ± 2522 MFI; trypsin: 36198 ± 5688 MFI). In-plate staining observed higher levels of ICAM-1 (58934 ± 2144 MFI) when compared to out-of-plate staining using trypsin (p <0.001).

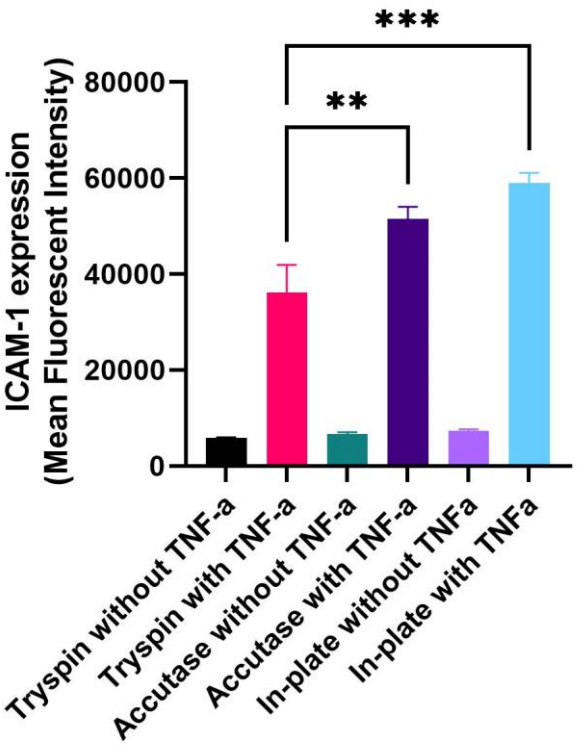


Figure 13. HCAEC expression of ICAM-1 (Mean Fluorescent Intensity) by out-of-plate staining using trypsin or accutase for detachmnet or in-plate staining, with and without endothelial cell activation by TNF-α. *Data shown are mean ± standard error mean (SEM). ***p <0.001, ****p <0.0001 for between group difference.*

The aim of this study was to determine an optimised method for analysis of endothelial adhesion molecule protein expression by flow cytometry. The current findings show that accutase does still cause some attenuation of adhesion molecules and therefore, the in-plate staining method remains superior compared to out-of-plate staining for detection of adhesion molecules by flow cytometry. However, accutase for cell detachment results in higher levels of HCAEC cell surface adhesion molecule protein expression when compared to using trypsin/EDTA, with a significant effect shown for ICAM-1 expression (**Figure 11A**).

Therefore, it is recommended to use accutase instead of trypsin/EDTA for cell detachment where in-plate staining is not possible. Our new findings show that the (Gräbner et al., 2000) method is the best method to prevent detachment-induced hydrolysation of CAM is to fix with formaldehyde and stain the endothelial monolayer before detaching the cells. Therefore, it is recommended that we use in-plate staining for detection of adhesion molecules. Hence, the method used throughout this thesis involves fixing the endothelial cell monolayer with formaldehyde then labelling with CAM mAbs, before detaching cells using accutase. The current data from TNF-α-induced endothelial cell activation also validates the flow cytometry mAbs for detection of CAM. Therefore, the method produced for detection of CAM was used with confidence for subsequent studies.

Appendix 9. Effect of 5-FU, epirubicin, cyclophosphamide, docetaxel, and FEC combined drug treatments on viability, annexin-V, and caspase-3 expression in HUVECs

		5-FU			
		3 hours			
Dosage (μM)	DMSO	0.0	1.0	1.5	2.0
Marker Expression					
Viability (%)	96.83±0.35	99.11±0.03	96.13±1.89	96.14±2.82	87.14±4.79
Caspase-3 (%)	4.43±3.07	8.98±1.07	10.67±1.640*	1.845±0.09*	0.86±0.05**
Annexin V (%)	3.33±0.35	18.37±2.32	22.54±1.92*	71.86±6.25****	72.39±10.54****
		12 hours			
Dosage (μM)	DMSO	0.0	1.0	1.5	2.0
Marker Expression					
Viability (%)	96.57±1.50	95.16±0.74	89.35±3.37	85.50±10.17	45.39±6.83***
Caspase-3 (%)	11.83±10.04	9.54±1.07	12.39±2.18	15.90±2.97	8.67±0.79
Annexin V (%)	18.43±15.36	14.74±2.58	16.51±3.96	77.23±2.94***	77.88±7.74***
		Epirubicin			
		4 hours			
Dosage (μM)	DMSO	0.0	0.003	0.006	0.009
Marker Expression					
Viability (%)	81.33±14.70	93.00±2.86	88.80±2.02	59.86±3.09*	58.50±1.60*
Caspase-3 (%)	9.23±7.02	4.21±0.25	13.31±0.67	46.79±2.33****	56.73±0.46****
Annexin V (%)	10.83±5.62	3.97±0.56	31.23±4.44**	48.57±3.35****	88.79±2.97****
		12 hours			
Dosage (μM)	DMSO	0.0	0.003	0.006	0.009
Marker Expression					
Viability (%)	95.23±2.73	98.84±0.50	95.77±1.10	96.41±1.22	92.66±2.82
Caspase-3 (%)	8.50±3.63	5.32±0.68	8.36±0.21	10.05±0.98	12.07±1.44*

Annexin V (%)	5.10±2.35	6.45±0.84	8.37±0.90	8.69±0.83*	11.56±1.96**
		Cyclophosphamide			
		3 hours			
Dosage (μM)	DMSO	0.0	19.0	38.0	57.0
Marker Expression					
Viability (%)	96.43±1.36	88.46±5.54	72.76±3.34***	62.92±0.28****	66.72±1.10***
Caspase-3 (%)	19.70±14.42	1.912±0.00	29.30±17.12	66.44±3.13*	66.10±4.48*
Annexin V (%)	13.00±13.89	2.214±0.00	31.41±21.40	73.16±3.69*	76.67±4.99*
		6 hours			
Dosage (μM)	DMSO	0.0	19.0	38.0	57.0
Marker Expression					
Viability (%)	92.23±5.37	94.74±1.76	84.64±0.63	68.87±3.12****	63.02±0.29****
Caspase-3 (%)	9.03±11.21	6.34±0.98	10.90±1.37	24.12±5.07	19.15±4.17
Annexin V (%)	12.33±14.29	8.76±1.08	14.07±1.74	24.12±5.07	22.18±4.67
		Docetaxel			
		24 hours			
Dosage (μM)	DMSO	0.0	3.0	6.0	9.0
Marker Expression					
Viability (%)	86.93±8.61	85.83±1.21	52.29±2.24***	57.24±4.78***	63.31±3.24**
Caspase-3 (%)	11.93±6.22	8.12±1.85	18.40±4.63	33.48±3.54*	14.73±6.82
Annexin V (%)	11.57±8.26	6.49±1.56	18.91±4.91	32.77±3.71*	13.28±5.28
		48 hours			
Dosage (μM)	DMSO	0.0	3.0	6.0	9.0
Marker Expression					
Viability (%)	80.70±5.56	87.06±1.55	48.01±2.70***	43.17±6.04***	51.32±5.12**
Caspase-3 (%)	31.20±6.35**	6.14±1.50	87.21±1.24****	81.00±1.49****	91.66±4.09****
Annexin V (%)	13.90±15.08	11.67±2.27	87.02±0.97****	84.88±1.44****	92.73±4.77****
		FEC			
		4 hours			

Dosage	DMSO	0.0	FEC 1	FEC 2	FEC 3
Marker Expression					
Viability (%)	92.23±3.50	98.92 ± 0.11	89.58±3.95	71.57±6.58*	55.15±5.80***
Caspase-3 (%)	3.73±0.18	3.56±0.58	15.26±5.11	49.13±10.09***	61.60±6.45***
Annexin V (%)	7.53±2.39	6.44±0.98	30.86±1.38	52.09±8.23***	80.98±7.75****
eNOS (MFC)	3325±329	3272 ±112	2593±789	2067±364	1087±371*
Phospho-eNOS (MFI)	4959±747	7462±938	6150±948	4782±601	5377±980
		12 hours			
Dosage	DMSO	0.0	FEC 1	FEC 2	FEC 3
Marker Expression					
Viability (%)	86.57±4.52	96.32±2.36	93.79±0.99	75.91±3.52	44.10±4.72****
Caspase-3 (%)	4.03±0.49	5.62±1.18	14.80±1.51	50.20±4.44****	68.31±7.74****
Annexin V (%)	8.73±2.63	5.12±0.72	8.73±1.52	45.49±17.28***	70.47±4.14****
eNOS (MFC)	7082±37	5207±984	5709±1293	1891±339**	1076±346**
Phospho-eNOS (MFI)	8944±963	8294±1428	9873±977	9510±1475	7918±666

*Data shown are mean ± SEM. *p <0.05, ** p<0.01, ***p<0.001, ****p<0.0001 between group difference in comparison to control group. FEC 1 = 5-FU: 1 µM, epirubicin: 0.003µM, cyclophosphamide: 19µM. FEC 2 = 5-FU: 1.5 µM, epirubicin: 0.006µM, cyclophosphamide: 38µM. FEC 3 = 5-FU: 2µM, epirubicin: 0.009µM, cyclophosphamide: 57µM. Mean Fluorescent Intensity (MFI).*

Appendix 10. HUVEC wound healing with exposure to 5-FU, epirubicin, cyclophosphamide, docetaxel, and FEC combined drug treatments.

5-FU	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-0.16±0.02		12.12±1.47		209.2±23.34	
Media	-0.15±0.02		11.72±2.02		198.8±40.59	
5-FU	-0.02±0.02		338.65±351.68		422.1±37.63	0.0006***
Epirubicin	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-0.16±0.02		11.15±0.37		203.4±19.43	
Media	-0.15±0.004		10.97±0.45		192.4±12.31	
Epirubicin	0.03±0.09		-426.71±759.16		605.9±192.9	0.009**
Cyclophosphamide	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-0.48333±		10.89±0.25		231.8 ±2.77	
Media	-0.45053		10.03±0.69		212±46.42	
Cyclophosphamide	-0.20463		19.75±1.25		496.5±89.45	0.010*
Docetaxel	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-0.08056		16.00±0.003		60.5±7.90	
Media	-0.05982		17.91±1.93		60.42±20.72	
Docetaxel	0.13383		-15.71±3.57		443.8±56.41	0.0005***
FEC	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-0.15662		11.15±0.37		203.4±19.43	
Media	-0.20098		11.60±0.78		269.2±77.12	
FEC	0.000747		-420.16±1491.90		277±40.35	0.024*

*Data shown are mean ± SEM. *p <0.05, ** p<0.01, ***p<0.001, ****p<0.0001*

Appendix 11. HCAEC viability, annexin-V, caspase-3, eNOS and Adhesion molecule expression post-exposure to FEC and docetaxel drug treatments.

		FEC			
		4 hours			
Dosage (μM)	DMSO	0.0	3.0	6.0	9.0
Marker Expression					
Viability (%)	96.00±0.95	96.77±0.69	93.93±1.43	89.17±1.36**	83.33±1.37****
Caspase-3 (%)	3.90±0.95	3.00±0.68	7.43±2.09	14.40±2.65*	24.47±1.47****
Annexin V (%)	15.40±3.49	8.37±2.09	26.13±6.03	40.23±4.59**	59.17±5.76****
eNOS (MFI)	2345±64.51	2243±96.84	2355±105.1	2709±217.2	2343±202.1
ICAM-1 (%)	1176 ± 120	2025 ± 274	988 ± 61**	423 ± 50***	431 ± 80 ****
		Docetaxel			
		24 hours			
Dosage	DMSO	0.0	FEC 1	FEC 2	FEC 3
Marker Expression					
Caspase-3 (%)	8.73±2.15	3.67±0.79	8.37±1.89	17.23±3.42**	5.87±0.85
Annexin V (%)	28.97±3.58	24.13±2.62	54.43±3.47***	67.90±3.95****	55.90±2.56***
eNOS (MFI)	3443±262.8	3918±145.0	2852±264.0	2748±259.7	2340±222.3*
ICAM-1 (MFI)	1746 ± 164	1849 ± 107	1155 ± 17**	990 ± 62***	877 ± 65 ***

*Data shown are mean ± SEM. *p <0.05, ** p<0.01, ***p<0.001, ****p<0.0001*

Appendix 12. HCAEC wound healing with exposure to 5-FU, epirubicin, cyclophosphamide, docetaxel, and FEC combined drug treatments.

5-FU	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-14.99±1.42		13.59±0.99		2498647 ±495176	
Media	-17.28±0.93		13.04±0.74		2677532±275732	
5-FU	0.036±0.19		-2860.43±5142.94		3393659±270561	0.025*
Epirubicin	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-15.06±1.42		13.62±0.98		2460814 ± 282797	
Media	-17.54±0.83		13.19±0.69		2665921± 148007	
Epirubicin	-5.98±5.75		-19.87±73.72		3470822 ± 292851	0.034*
Cyclophosphamide	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-15.33±1.54		12.86±0.82		2557768±664147	
Media	-17.69±0.99		12.17±0.34		2831506±216759	
Cyclophosphamide	-9.79±2.40		24.54±8.44		3413935±306531	0.056
Docetaxel	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-8.17±1.14		17.42±1.34		1452484±340092	
Media	-8.96±0.56		16.83±1.19		1505577±176978	

Docetaxel	-1.07±0.50		231.38±159.49		3123411±309286	0.002**
FEC	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-15.06±1.42		13.62±0.98		2460814±489819	
Media	-17.54±0.83		13.19±0.70		2665921±256356	
FEC	-1.02±0.62		381.60±365.77		3786777±787307	0.034*

*Data shown are mean ± SEM. *p <0.05, ** p<0.01, ***p<0.001, ****p<0.0001*

Appendix 13. Effects of active vs inactive serum preconditioning on HUVEC viability, annexin-V, caspase-3 and eNOS expression with exposure to 5-FU, epirubicin, cyclophosphamide, docetaxel, and FEC combined drug treatments.

5-FU			
3 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Viability (%)	87.99±2.94	96.51±1.73	0.033*****
Caspase-3 (%)	14.53±1.45	13.25±1.03	0.286
Annexin V (%)	74.23±4.24	56.22±6.78	0.044*
12 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Viability (%)	66.13±2.87	83.50±7.49	0.048*****
Caspase-3 (%)	11.71±1.44	12.96±1.95	0.316
Annexin V (%)	74.66±1.37	77.02±2.09	0.200
Epirubicin			
4 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Viability (%)	66.02±3.05	72.64±5.40	0.173
Caspase-3 (%)	30.76±1.03	29.93±5.56	0.445
Annexin V (%)	18.95±1.65	31.73±8.08	0.098
12 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Viability (%)	96.08±2.78	96.11±0.29	0.496
Caspase-3 (%)	7.56±1.11	2.15±0.17	0.004******
Annexin V (%)	5.95±1.24	3.06±0.26	0.042*****
Cyclophosphamide			
3 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Viability (%)	62.80±1.86	63.99±0.64	0.577
Caspase-3 (%)	68.02±2.33	60.52±5.73	0.146
Annexin V (%)	69.66±5.87	77.37±1.97	0.141
6 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Viability (%)	64.92±5.31	63.14±7.34	0.427
Caspase-3 (%)	14.97±2.28	11.60±1.09	0.127
Annexin V (%)	18.42±2.22	14.15±1.05	0.079
Docetaxel			
24 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Viability (%)	71.19±2.38	68.35±2.49	0.228

Caspase-3 (%)	12.74±1.62	12.54±1.02	0.459
Annexin V (%)	11.83±1.57	11.32±0.98	0.398
48 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Viability (%)	61.23±5.19	54.83±4.18	0.196
Caspase-3 (%)	47.21±2.95	45.86±3.13	0.385
Annexin V (%)	58.02±2.95	55.59±3.01	0.297
FEC			
4 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Viability (%)	81.41±1.13	82.34±1.68	0.334
Caspase-3 (%)	47.37±5.97	48.04±8.80	0.476
Annexin V (%)	55.39±5.04	44.88±7.48	0.144
eNOS (MFI)	7479±3139	9914±4421	0.338
Phospho-eNOS (MFI)	5217±987	6093±2015	0.358
12 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Viability (%)	92.14±1.85	91.24±3.43	0.413
Caspase-3 (%)	55.92±9.99	58.40±2.75	0.409
Annexin V (%)	63.91±8.80	62.52±6.34	0.451
eNOS (MFI)	18132±253	18167±211	0.460
Phospho-eNOS (MFI)	8046±824	7730±966	0.408

*Data shown are mean ± SEM. *p <0.05.*

Appendix 14. Exercise training effect on HUVEC wound healing with exposure to 5-FU, epirubicin, cyclophosphamide, docetaxel, and FEC combined drug treatments.

5-FU	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-0.16±0.02		12.12±1.47		209.2±23.34	
Media	-0.15±0.02		11.72±2.02		198.8±40.59	
5-FU	-0.02±0.02		338.65±351.68		422.1±37.63	
5-FU + inactive	-0.004±0.01		126.71±641.76		387.1±29.8	
5-FU + active	-0.02±0.01	0.08	169.98±100.88	0.46	430.7±157.4	0.66
Epirubicin	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-0.16±0.02		11.15±0.37		203.4±19.43	
Media	-0.15±0.004		10.97±0.45		192.4±12.31	
Epirubicin	0.03±0.09		-426.71±759.16		605.9±192.9	
Epi + inactive	0.07±0.03		-29.32±17.15		386±9.74	
Epi + active	0.01±0.12	0.27	-21.52±43.65	0.39	399.2±156.2	0.89
Cyclophosphamide	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-0.48333±		10.89±0.25		231.8 ±2.77	
Media	-0.45053		10.03±0.69		212±46.42	
Cyclophosphamide	-0.20463		19.75±1.25		496.5±89.45	
Cyc + inactive	-0.15492		15.91±1.95		399.7±124.6	
Cyc + active	-0.09023	0.27	17.28±5.87	0.36	287.8±47.95	0.22
Docetaxel	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-0.08056		16.00±0.003		60.5±7.90	
Media	-0.05982		17.91±1.93		60.42±20.72	
Docetaxel	0.13383		-15.71±3.57		443.8±56.41	
Doc + inactive	0.061203		-82.40±116.70		260.9±48.56	

Doc + active	-0.00242	0.09	40.72±57.25	0.09	212±23.23	0.019*
FEC	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-0.15662		11.15±0.37		203.4±19.43	
Media	-0.20098		11.60±0.78		269.2±77.12	
FEC	0.000747		-420.16±1491.90		277±40.35	
FEC + inactive	0.008411		-215.67±115.02		292.5±86.06	
FEC + active	-0.0042	0.009***	359.28±148.57	0.003***	280±115.3	0.89

*Data shown are mean ± SEM. *p <0.05.*

Appendix 15. Physical activity effect on HCAEC viability, annexin-V, caspase-3, eNOS and Adhesion molecule expression post-exposure to FEC and docetaxel drug treatments.

FEC			
4 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Caspase-3 (%)	12.87±1.77	12.83±1.47	0.495
Annexin V (%)	36.33±5.38	32.90±3.59	0.312
eNOS (MFI)	2012±120.7	1852±94.51	0.177
ICAM-1 (MFI)	399±35	711±53	0.004**
Docetaxel			
24 hours			
Marker Expression	Inactive	Active	Between group difference (*P* Value)
Caspase-3 (%)	6.90±1.80	5.43±1.68	0.292
Annexin V (%)	48.97±2.60	39.63±2.70	0.034*
eNOS (MFI)	2527±140.3	3031±148.5	0.034*
ICAM-1 (MFI)	396±37	693±56	0.006**

*Data shown are mean ± SEM. *p <0.05.*

Appendix 16. Physical activity serological conditioning effect on HCAEC wound healing with exposure to 5-FU, epirubicin, cyclophosphamide, docetaxel, and FEC combined drug treatments.

5-FU	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-14.99±1.42		13.59±0.99		2498647±495176	
Media	-17.28±0.93		13.04±0.74		2677532±275732	
5-FU	0.036±0.19		-2860.43±5142.94		3393659±270561	
5-FU + inactive	5.96±5.85		-111.85±126.94		3778641±693937	
5-FU + active	-1.01±0.44	0.125	227.76±156.30	0.022*	3235360±273396	0.137
Epirubicin	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-15.06±1.42		13.62±0.98		2460814±282797	
Media	-17.54±0.83		13.19±0.69		2665921±148007	
Epirubicin	-5.98±5.75		-19.87±73.72		3470822±292851	
Epi + inactive	-13.19±2.71		17.02±5.31		2604209±399530	
Epi + active	-2.78±0.41	0.003**	65.67±14.91	0.003**	3021422±199032	0.18

Cyclophosphamide	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-15.33±1.54		12.86±0.82		2557768±664147	
Media	-17.69±0.99		12.17±0.34		2831506±216759	
Cyclophosphamide	-9.79±2.40		24.54±8.44		3413935±306531	
Cyc + inactive	-9.81±6.35		79.77±112.58		2994794±440549	
Cyc + active	-7.24±6.38	0.354	-23.06±72.36	0.127	3266762±317608	0.435
Docetaxel	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-8.17±1.14		17.42±1.34		1452484±340092	
Media	-8.96±0.56		16.83±1.19		1505577±176978	
Docetaxel	-1.07±0.50		231.38±159.49		3123411±309286	
Doc + inactive	-0.806±1.45		455.52±1021.16		3373108±192992	
Doc + active	-3.32±0.30	0.037*	59.17±6.18	0.271	2950014±30542	0.009**
FEC	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
DMSO	-15.06±1.42		13.62±0.98		2460814±489819	

Media	-17.54±0.83		13.19±0.70		2665921±256356	
FEC	-1.02±0.62		381.60±365.77		3786777±787307	
FEC + inactive	0.88±1.81	0.300	-137.14±355.84	0.495	3873552±389449	0.275
FEC + active	0.14±0.36		-142.06±587.08		3442727±443050	

*Data shown are mean ± SEM. *p <0.05.*

Appendix 17. Acute exercise training effect on HCAEC Annexin-V, caspase-3, eNOS and Adhesion molecule expression post-exposure to FEC and docetaxel drug treatments.

FEC			
4 hours			
Marker Expression	Pre	Post	*P* Value
Annexin V (%)	45.3±1.47	39.2±1.42	0.020*
Caspase-3 (%)	8.3±0.59	8.97±2.15	0.363
eNOS (MFI)	2054±85.25	1919±112.3	0.068
ICAM-1 (MFI)	1083 ± 86	1207 ± 75	0.126
Docetaxel			
48 hours			
Marker Expression	Pre	Post	*P* Value
Annexin V (%)	69.9±1.97	75.2±8.20	0.276
Caspase-3 (%)	5.5±0.81	4.03±0.24	0.117
eNOS (MFI)	4319±304.7	4484±418.1	0.370
ICAM-1 (MFI)	735 ± 28	862 ± 37	0.012****

Appendix 18. Acute exercise training effect on HCAEC wound healing with exposure to 5-FU, epirubicin, cyclophosphamide FEC combined, and docetaxel drug treatments.

5-FU	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
Pre No drug	-14.48±1.77		11.10±0.74		1965113±391576	
Post No drug	-13.35±0.46		12.65±0.02		1959433±97367	
Pre Drug	-0.46±0.71		65.95±556.32		3867441±297728	
Post Drug	-3.63±2.72	0.113	131.67±131.28	0.442	3706101±656248	0.694
Epirubicin	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
Pre No drug	-13.51±5.85		11.91±12.51		1403627±494092	
Post No drug	-13.49±5.86		8.27±7.62		1918809±104488	
Pre Drug	-10.14±5.20		13.32±13.11		2729685±241830	
Post Drug	-12.15±5.26	0.233	11.58±11.58	0.335	2871750±296381	0.644
Cyclophosphamide	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
Pre No drug	-12.75±1.27		16.08±6.85		1706844± 502332	
Post No drug	-13.52±0.54		12.52±0.03		2196285±115076	
Pre Drug	-6.66±5.68		-136.33±274.01		2637136±312925	
Post Drug	-13.69±1.19	0.130	15.61±1.69	0.218	2743462±282441	0.471
FEC	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**

Pre No drug	-12.73±1.08		15.85±7.22		1403627±494092	
Post No drug	-13.34±0.51		12.66±0.05		1918809±104488	
Pre Drug	3.47±4.64	0.204	-1468.79±2060.13	0.133	4610024±1476312	0.496
Post Drug	-0.19±0.96		93.61±297.16		3786322±794717	
Docetaxel	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
Pre No drug	-5.18±1.10		23.10±16.47		898039±218133	
Post No drug	-6.53±0.30		15.72±0.18		1046524±66146	
Pre Drug	0.76±1.63		-18.78±121.59		2919327±1081240	
Post Drug	-2.84±1.87	0.095	109.10±97.64	0.169	2639901±781689	0.778

Appendix 19. Exercise training intervention effect on HCAEC Annexin-V, cleaved caspase-3, eNOS and Adhesion molecule expression post-exposure to FEC and docetaxel drug treatments.

FEC			
4 hours			
Marker Expression	Pre	Post	*P* Value
Annexin V (%)	42.23±4.02	29.57±4.10	0.046*
Caspase-3 (%)	6.13±0.50	4.43±0.30	0.010*
eNOS (MFI)	1612±163.0	1854±77.34	0.250
ICAM-1 (MFI)	1030 ± 113	1105 ± 63	0.353
Docetaxel			
48 hours			
Marker Expression	Pre	Post	*P* Value
Annexin V (%)	78.70±2.95	69.37±5.98	0.106
Caspase-3 (%)	8.30±1.66	7.33±1.08	0.359
eNOS (MFI)	4386±218.0	4123±163.5	0.256
ICAM-1 (%)	1005 ± 150	931 ± 47	0.375

Appendix 20. Exercise training intervention effect on HCAEC wound healing with exposure to 5-FU, epirubicin, cyclophosphamide FEC combined, and docetaxel drug treatments.

5-FU	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
Pre No drug	-16.23±0.69		13.30±0.25		2527388±173230	
Post No drug	-12.45±1.07		12.77±0.82		1854255±317802	
Pre Drug	-3.49±1.97		123.22±119.85		4086790±72739	
Post Drug	-10.18±5.39	0.163	28.76±15.67	0.170	3293127±318612	0.031*
Epirubicin	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
Pre No drug	-16.42±0.61		13.44±0.15		2512912±153174	
Post No drug	-12.50±1.15		12.83±0.91		1827683±331488	
Pre Drug	-14.23±5.97		22.55±14.21		3193470±410646	0.572
Post Drug	-16.64±1.78	0.341	15.44±1.13	0.249	2987184±203927	
Cyclophosphamide	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
Pre No drug	-33.14±1.48		7.50±0.09		2736123±148374	
Post No drug	-25.23±2.27		7.40±0.35		2057433±312904	
Pre Drug	-22.72±8.22		10.31±3.90		2457098±153259	
Post Drug	-27.58±0.94	0.260	7.98±0.22	0.215	2434208±101016	0.714
FEC	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
Pre No drug	-16.42±0.61		13.44±0.15		2512912±153174	
Post No drug	-11.64±1.07		12.23±0.72		1620242±293942	

Pre Drug	-0.67±0.05	0.085	255.74±22.84	0.107	3070323±38504	0.153
Post Drug	0.20±0.53		-95.18±361.62		3256372±104679	
Docetaxel	**$V_{migration}$ (µm/hr)**	**P Value**	**$T_{1/2gap}$ (hours)**	**P Value**	**Area under curve**	**P value**
Pre No drug	-8.25±0.40	0.022 *	16.95±0.33	0.311	1392071±102621	0.194
Post No drug	-5.60±0.67		14.91±1.30		871485±181777	
Pre Drug	-0.36±0.33		-318.96±1054.91		2998166±77529	
Post Drug	-5.21±1.82		41.26±24.99		2276122±572130	

Appendix 21.

Protecting vascular endothelial cells against breast cancer chemotherapy toxicity using serological preconditioning from exercise-trained women

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PURPOSE: To determine if there are protective effects of habitual physical activity on chemotherapy-induced vascular toxicity.

METHODS: Human umbilical vein endothelial cells (HUVECs) were cultured with 5% serum from healthy trained (n=3) and untrained (n=3) females. After 24 hours of serum preconditioning, HUVECs were exposed to physiological concentrations of 5-fluorouracil (5-FU), epirubicin, cyclophosphamide, and docetaxel (FEC-T). At 12 hours, the effects of exercise and chemotherapy on HUVEC viability and apoptosis were measured using intracellular flow cytometry. To assess endothelial repair, the preconditioned endothelial monolayer was scraped and exposed to FEC-T drugs. Wounds were imaged at 0, 3, 4, 6, 12, 24, and 48 hours to determine rate of gap closure.

RESULTS: FEC-T chemotherapy drugs decreased viability of HUVECs by 67±6%, increased caspase-3 activity by 78±4% and annexin-V expression by 88±3% (all p <0.05), compared to no drug control. Preconditioning HUVECs with serum from exercise trained females prior to chemotherapy exposure resulted in improved viability of HUVECs, and a reduction in caspase-3 and annexin-V expression compared to preconditioning with serum from untrained females (5-FU: viable HUVECs for active: 97±2% vs sedentary: 88±3%, p=0.03; phosphatidylserine expression for active: 56±7% vs sedentary: 74±4%, p=0.04; epirubicin: caspase-3 expression for active: 2±1% vs sedentary 8±1%, p=0.04; phosphatidylserine expression for active: 5±1% vs sedentary: 9±2%, p=0.03). FEC-T also resulted in impaired HUVEC wound healing by 100±1% (p=0.01). Preconditioning HUVECs with serum from trained individuals attenuated this impairment favourably over serum from untrained individuals (untrained $V_{migration}$ impairment: 10±4% vs trained $V_{migration}$ impairment: 4±1%, p=0.02).

CONCLUSION: Our results confirm that FEC-T drugs commonly used in breast cancer treatment cause apoptosis the disruption of endothelial cell repair. Chemotherapy-induced endothelial cell apoptosis and impaired wound healing may be attenuated in exercise trained individuals.