Mitigation of Anthracycline Cardiotoxicity & Cardiovascular Risk Profiling of Cancer Patients

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Abstract

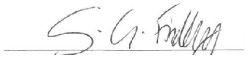
Anthracyclines remain one of the most widely used classes of cancer chemotherapy with proven efficacy across a wide range of malignancies. Despite clear anti-tumour efficacy in both adults and children, anthracyclines are attributed with high rates of 'cardiotoxicity'. Anthracycline-induced cardiotoxicity (AIC) is frequently undiagnosed and subsequently presents as symptomatic cardiac failure, conveying a poor prognosis. Therefore, when considering cancer patients, many of whom already have a higher baseline risk of cardiovascular disease compared with the general population, AIC threatens to limit long-term cancer patient survival.

Various drug infusion rates, newer formulations, and more recently Angiotensin Converting Enzyme inhibitors (ACEi) have been administered to prevent late-onset cardiotoxicities, whilst maximising anthracycline efficacy. Despite ACEi being well studied in cardiovascular medicine, their mechanism(s) for the treatment and prevention of AIC remains unknown. The study objective was to investigate the relationship between AIC and angiotensin signalling mechanisms, in both preclinical and clinical studies. Exposure of the AC10 human ventricular cardiomyocyte cell line to clinically relevant concentrations of doxorubicin *in vitro* showed a significant concentration- and time-independent induction of angiotensin II receptor type 1 (AT1R) gene expression. This supports previous observations that doxorubicin induces cellular hypertrophy in AC10 cells, an effect mitigated by exposure to angiotensin receptor blocking therapeutics. Regarding clinical effects, the study also demonstrates a relationship between anthracycline-induced cardiotoxicity and genotype of ACE, presence of cellular hypertrophy and fibrosis in cardiac tissue of patients treated with doxorubicin, and baseline blood pressure and subsequent development of AIC.

These findings are significant in offering a new paradigm for the mechanism of AIC and protective strategies for its identification and mitigation. This research therefore supports interference with angiotensin signalling as a putative clinical strategy, supporting the transition towards preventative strategies for cardiotoxicity, advocating a proactive approach to cardiovascular risk assessment, and minimising risks through primary prevention.

Declaration

I declare that this thesis entitled 'Mitigation of Anthracycline Cardiotoxicity & Cardiovascular Risk Profiling of Cancer Patients' is original research, which I have undertaken for the award of Doctor of Philosophy. I hereby confirm that this thesis has not been submitted for a higher degree to any other University or Institution, and that there is no plagiarism contained within, with all sources of information cited and appropriately referenced.



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List of abbreviations

5-FU	5-Fluorouracil
AIC	Anthracycline-induced cardiotoxicity
AC10-CMs	AC10 cardiomyocytes
ACE	Angiotensin converting enzyme
AR	Angiotensin Receptor
ARB	Angiotensin receptor blocker
AT1R	Angiotensin II type-1 receptor
BSE	British Society of Echocardiography
CHF	Congestive heart failure
DAPI	4’,6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's medium F-12 Ham
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetatraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
GLS	Global longitudinal strain
HBSS	Hanks’ Balanced Salt Solution
hiPSC	Human induced-pluripotential stem cells
hiPSC-CM	Human induced-pluripotential stem cell derived cardiomyocytes
HPLC	high-performance liquid chromatograph
HPRT	Hypoxanthine guanine phosphoribosyl transferase
hsCRP	High-sensitivity C-reactive protein
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-mass spectrometry with tandem mass spectrometry
LVF	Left ventricular failure
LVEF	Left ventricular ejection fraction
LVH	Left ventricular hypertrophy
M-MLV	Moloney Murine Leukaemia Virus

MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
MUGA	Multi-gated acquisition
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative PCR
QUOD	Quality in Organ Donation
RAAS	Renin-angiotensin-aldosterone system
RIA	Radioimmunoassay
RPL13A	60s ribosomal protein L13A
Rpm	Revolutions per minute
RT-PCR	Reverse-transcriptase polymerase chain reaction
RTCA	Real-time cell analyser
SDS	Sodium Dodecyl Sulphate
SiRNA	Short interfering RNA
SPE	Solid phase extraction
TAE	Tris, Acetic Acid, EDTA Buffer
TBP	TATA-binding protein
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline 0.1% Tween
VAD	Ventricular assist device

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Chapter 1. Introduction

1.1 Relationship between cancer and cardiovascular disease

Following considerable advances to cancer treatments and care, the number of cancer survivors continues to rise, with an estimated 2.5 million cancer patients living within the United Kingdom (UK) (Maddams et al., 2012). These improvements in mortality rates have also been observed on a global scale, with worldwide survivorship statistics demonstrating that despite the increased cancer incidence, 5-year survival has significantly improved over the past two decades (Allemani et al., 2015, Bray et al., 2018, Ferlay et al., 2018). With the improvements in both cancer prognosis and earlier diagnosis, cancer survivors are living longer however, this means that with advancing age, they are more susceptible to cardiovascular disease.

Cancer and cardiovascular disease have an interesting interdependent relationship, especially within the UK, where cardiovascular disease is the second most common cause of mortality after cancer (Bhatnagar et al., 2016). With the marked improvements in overall cancer prognosis, cancer survivors are experiencing prolonged exposure to cardiovascular risk factors, therefore contributing to cardiovascular morbidity and mortality (Mulrooney et al., 2009, Patnaik et al., 2011). Similarly, major advances in cardiovascular care have seen a dramatic reduction in patient mortality from coronary heart disease, heart failure and other acute cardiovascular events. Their improved life expectancy therefore exposes these patients to an increased risk of cancer throughout their lifetime (Bhatnagar et al., 2016).

After secondary malignancies, cardiovascular disease is the leading cause of mortality amongst cancer survivors (Wilson et al., 2017). Cardiovascular risk factors have been well studied, with their presence having a significant impact upon a patient's lifetime risk of developing cardiovascular disease. Recent studies have indicated that cancer survivors are exposed to a higher risk of developing cardiovascular disease than the general population, and whilst these findings may be attributed to cumulative co-morbidities, the toxic effects of cancer therapy has been shown to have a significant impact upon cancer patients' risk of cardiovascular events and long-term survival (Strongman et al., 2019, Paterson et al., 2022). As cancer survival rates continue to improve, together with the application of novel therapies and enhanced detection of cardiovascular toxicity, the significance of 'cardiotoxicity' has seen the development of

cardio-oncology as a clinical sub-specialty. The incorporation of cardiology care and guidelines within the holistic approach to cancer care aims to maximise patients' quality of life during their cancer care and beyond, through evidence-based prevention of cardiovascular morbidity and mortality.

1.2 *Cardio-oncology and cardiotoxicity*

Cardiovascular toxicity is now one of the most frequent adverse effects of cancer therapy, recognised as adverse changes to cardiac structure and/or function due to cancer treatment (Lyon et al., 2022). Radiotherapy-induced cardiovascular toxicity is primarily associated with the development of coronary artery disease through endothelial dysfunction and atherogenesis, whereas chemotherapy-induced toxicity exhibits a broad range of cardiac effects ranging from acute effects such as cardiac arrhythmias, through to later chronic effects including hypertension, and myocardial dysfunction, in addition to coronary artery disease (Zamorano et al., 2020). In terms of chronic cardiotoxicity, this is often described synonymously with cardiomyopathy, however, it is also represented as cardiac damage due to cancer treatment. The diagnosis of chronic cardiovascular toxicity represents a challenge due to its insidious onset, presenting at least one year after therapy and even extending up to decades beyond completion of cancer treatment (Curigliano et al., 2012). Historically, cardiotoxicity of oncology agents was classified as either acute, early-onset chronic (occurring within one year post-treatment) or late-onset chronic (developing years or decades after treatment) (Curigliano et al., 2012). However, it is now believed that cardiotoxicity is actually a continuous process, initiated at the acute cellular damage level and subclinical myocardial injury, followed by asymptomatic progressive functional decline, which can culminate in symptomatic left ventricular systolic failure, the latter having a high mortality rate (Henriksen, 2018, Cardinale et al., 2020). As clinical outcomes and patient prognosis from cancer therapy continue to improve, a new concern for clinicians is how to prevent these cardiotoxic effects and identify patients at risk, especially given the impact upon cancer treatment selection and long-term patient survival (Muhandiramge et al., 2022).

With the advancement of novel cancer treatments and prolonged survival, prevention of cardiovascular morbidity and mortality has never been more important. The progression of cardio-oncology as a clinical sub-speciality has seen a rapidly expanding translational research area which focusses on establishing vital prophylactic and cardiotoxicity management strategies. By enhancing our awareness and understanding of the mechanisms of cancer

therapy-induced toxicities, this research seeks to develop earlier detection of these cardiovascular events whilst simultaneously identifying approaches to reduce overall cardiovascular risk within this patient population.

1.2.1 Drug-induced cardiotoxicity of cancer therapies

The definition of drug-induced cardiotoxicity encompasses a range of adverse effects upon the cardiovascular system, including electrophysiological disturbances and arrhythmias, circulatory effects such as coronary artery disease, and arterial/venous thromboembolism, in addition to structural effects upon the heart culminating in hypertension and ultimately heart failure. This cardiotoxicity has been described as either irreversible or reversible, dependent upon the mode of action being either cytotoxic, cytostatic or perturbation of cellular physiological processes. Irreversible cardiotoxicity is exemplified by anthracyclines, resulting in initial death of cardiomyocytes and other cardiac cell types, and thereafter progression to cardiac failure. This is distinguishable from reversible drug-induced cardiotoxicity, associated with drugs such as the monoclonal antibody trastuzumab and kinase inhibitors, which cause physiological rather than pathological changes and preserve cellular structural integrity (Ewer and Ewer, 2015). In this latter case, the therapeutics function to disrupt or inhibit signalling pathways associated with cell proliferation and response to growth factors combined with other extracellular factors, which retard tumour growth per se, rather than induce direct cytotoxic effects and myocyte injury. Cardiotoxicity with these agents is often reversed by cessation of drug treatment or changes to drug administration (Ewer and Ewer, 2015).

Reversible and acute drug-induced cardiotoxicities are commonly identified by electrophysiological and cardiac functional changes. In contrast, the diagnosis of irreversible and chronic cardiotoxicity is confirmed through a combination of biochemical markers (e.g. increases in systemic troponin levels) and functional classifications (e.g. a reduction in LVEF of ≥10% from baseline to a value of <53%), which are well established within international clinical guidelines (Plana et al., 2014, Zamorano et al., 2017, Dobson et al., 2021, Lyon et al., 2022).

1.3 Anthracycline-induced cardiotoxicity

Anthracycline chemotherapy (*e.g.* daunorubicin, doxorubicin, epirubicin and idarubicin) remains one of the most widely used cancer treatments with proven efficacy across a wide range of solid tumours and haematological malignancies, notably lymphoma, leukaemia, breast cancer and sarcoma. However, despite clear anti-tumour efficacy in both adults and

children, anthracycline chemotherapy is attributed with a high rate of 'cardiotoxicity' (McGowan et al., 2017).

Anthracyclines exert their anti-neoplastic effects through DNA intercalation, intracellular oxidative stress and inhibition of topoisomerase II activity, impairing subsequent DNA replication and culminating in cell growth inhibition and cell death (Barry et al., 2007). Although anthracyclines exert an anti-proliferative effect upon the cancer cells, their high intracellular potency and accumulation unintentionally exposes myocardial cells to their toxic effects. The ensuing myocyte death and limited regeneration capabilities of damaged cardiac tissue progresses towards overt cardiac failure, which is frequently the stage when cardiotoxicity is first detected in the clinic. Consequently, anthracycline-induced cardiotoxicity (AIC) often remains undiagnosed until patients present symptomatically several years post-treatment. Therefore, when considering cancer patients, many of whom already have a higher baseline risk of cardiovascular disease compared with general population, anthracycline-induced cardiotoxicity threatens to limit long-term cancer patient survival (Patnaik et al., 2011).

Anthracycline-induced cardiotoxicity was first described in 1971, within the clinical trials of Adriamycin (doxorubicin). Despite their success as a highly effective anti-tumour therapy, it was recognised that high anthracycline cumulative doses were associated with severe stages of cardiovascular toxicity (Middleman et al., 1971). Determining the full extent of anthracycline-induced cardiotoxicity within the cancer survivor population remains a complex challenge for clinicians. Acute cardiotoxicity, although rare (<1%), is readily recognisable through its close association with intravenous infusions or immediately after completion of an anthracycline chemotherapy cycle, with clinical effects usually reversible. Typically this will present acutely as a cardiac arrhythmia, hypotension, or in extreme cases as left ventricular failure, myocarditis or pericarditis (Takemura and Fujiwara, 2007). Histologically, the myocardial infiltrates are composed of neutrophils and lymphocytes, giving rise to a predominantly inflammatory response, which therefore accounts for the high degree of reversibility as patients are managed supportively and demonstrate good recovery (Berry and Jorden, 2005).

The diagnosis of chronic anthracycline-induced cardiotoxicity is rather more complex, given the latency of presentation combined with the subtleness of myocardial injury, and relative asymptomatic nature of its development. Although the estimated early-incidence of cardiac

failure ranges from 1 to 16%, it is likely that the prevalence chronic cardiotoxicity is severely underestimated due to the progressive nature and pathophysiology of chronic cardiotoxicity (Barry et al., 2007). Late-onset chronic progressive cardiotoxicity, associated with cardiomyopathy presenting more than one year post-anthracycline chemotherapy, is recognised in both children and adults, however, their respective pathological processes are believed to take different courses due to the varying cellular developmental states and cardiac stresses (Lipshultz et al., 2008). Adult patients receiving anthracycline chemotherapy may experience initial cardiomyocyte damage resulting in a combination of impaired cardiomyocyte hyperplasia and death of existing cardiac cells. The consequences of this are reflected by the studies of Olvetti et al. focused on understanding the development processes of the adult heart, in which the continual loss of cardiomyocytes in adult patients was shown to be at a rate of around 50 million myocytes per year (Olivetti et al., 1991). This loss being compensated by early growth regulated by myocyte hypertrophy together with interstitial fibroblast hyperplasia. Although this stimulated an early increase in myocyte cell volume, the overall loss of muscle mass and subsequent lack of myocardial stretch exposed older patients to cardiac failure, due to loss of the normal compensatory capacity of the heart (Olivetti et al., 1991). Therefore, in the case of anthracycline-induced cardiotoxicity and cellular loss, the remaining cardiomyocytes are able to compensate, predominantly through hypertrophy (Olivetti et al., 1991). However, cardiomyocyte fibrosis and ventricular wall thinning soon ensue, rendering these adaptive mechanisms insufficient to cope with the increased left ventricular (LV) afterload (Bernaba et al., 2010). Therefore, a reduction in left ventricular function develops and ultimately a dilated cardiomyopathy, the hallmark of advanced cardiotoxicity (Lipshultz et al., 2005). It is often at this late stage when patients first present to the cardio-oncology clinic, sometimes precipitated by high cardiac output states such as sepsis, pregnancy, surgery, or anaemia, as the damaged heart is unable to adequately compensate, and unfortunately this presentation conveys a high mortality rate, up to 50% five-year mortality rate, unless cardiac transplantation is a viable treatment option (Cardinale et al., 2010, Taylor et al., 2019).

1.3.1 *Paediatric anthracycline-induced cardiotoxicity*

Cardiotoxicity in paediatric patients displays many similarities to that seen in adults, particularly in the latter stages of the development of anthracycline-induced cardiomyopathy. However, the paediatric heart has been exposed to lower levels of cardiac stresses and has not

yet reached the maximal cellular mass associated with adult cardiac tissue (Mollova et al., 2013). It is feasible that damage through direct anthracycline toxicity may result in paediatric patients' hearts never achieving normal myocyte numbers, in addition to limiting their regeneration capabilities. Long-term studies of anthracycline-induced cardiotoxicity appear to confirm this, with paediatric cancer survivors displaying a significant decrease in LV wall mass, thickness and compliance (Wojtacki et al., 2000). Therefore, when these patients reach cardiac maturity, they already lack the haemodynamic compensatory mechanisms to withstand myocardial stress and reach the diagnostic threshold of left ventricular dysfunction at an earlier stage. Lipshultz and colleagues have compiled extensive research evaluating cardiac echocardiographic changes in paediatric cancer survivors. They concluded that cardiotoxicity in paediatric patients may transition from an early subclinical dilated cardiomyopathy towards a more restrictive cardiomyopathy presentation, mediated by the reduction in left ventricular mass and cardiac chamber size ('Grinch syndrome') (Lipshultz et al., 2014). Cardiac imaging studies have progressed this theory with comprehensive evaluation of LV mass pre- and post-anthracycline chemotherapy to predict cardiovascular outcomes in cancer patients (Jordan et al., 2018). In response to this appreciation of cardiovascular physiology and advanced imaging detection, physicians believe that effective prevention of cardiotoxicity mediated through anthracycline administration, cardiovascular risk factor modification and cardioprotective agents is the key to improving patients' long term survival following their cancer treatment (Zhang et al., 2016). Given these explained physiological differences between paediatric and adult cardiomyocytes, the mechanisms of paediatric and adult anthracycline-induced cardiotoxicity are currently recognised as separate entities. Therefore, the evaluation of anthracycline-induced cardiotoxicity in paediatric patients was considered outside of the scope of this thesis and will be pursued in future translational research projects.

1.3.2 *Dose-related cardiotoxicity of anthracyclines*

Several pharmacological studies have focused on the identification of factors associated with development of anthracycline-induced cardiotoxicity, primarily for doxorubicin. After intravenous administration to patients, doxorubicin displays an initial fast distributive half-life of 3-5 minutes, suggesting rapid tissue uptake, and a slow elimination phase, reflected by a terminal pharmacokinetic half-life of between 20-30 hours (Eksborg, 1990, Barpe et al., 2010). This, in combination with a steady-state distribution volume of 810-1200 L/m^2 is indicative of extensive drug uptake into tissues, including cardiac tissue, giving rise to a range of inadvertent

toxicities. The cumulative dose of anthracycline therapy has been shown to have a strong bearing upon the incidence of cardiotoxicity, with higher doses associated with increased cellular damage and development of dilated cardiomyopathy (Swain et al., 2003). The major risk factor for development of doxorubicin-induced cardiac dysfunction was found to be the cumulative drug exposure, with the incidence of clinical heart failure reported to rise exponentially from 5% with a cumulative dose of 400 mg/m^2 to 48% with 700 mg/m^2 (Henriksen, 2018). This risk was augmented in older patients, especially when administered as part of multi-modality or multi-agent therapeutic approaches (Lotrionte et al., 2013, Jiang et al., 2018). However, despite limiting total cumulative doxorubicin exposure to 450 mg/m^2 which resulted in fewer acute cardiovascular toxicity events (Cappetta et al., 2018), no significant reduction in late-onset complications was confirmed, implicating that no dose of anthracycline is inherently safe for the cardiovascular system (Lipshultz et al., 2005, Cappetta et al., 2018). This research was enhanced by Lipshultz and colleagues through long-term follow-up of acute lymphoblastic leukaemia patients, also demonstrating that even patients exposed to the lowest doses of anthracyclines are at risk of severe cardiac failure, whilst those treated with the highest doses of chemotherapy may never develop clinical signs of cardiotoxicity (Nysom et al., 1998, Lipshultz et al., 2005). Various infusion rates (bolus, short infusions of ≤ 1 hour, or extended continuous infusions of up to 96-hours) and newer formulations (analogues, pegylation, liposomes and other nanodelivery systems) have been trialled, aiming to reduce the peak plasma concentrations (Cmax) and limit toxic side effects, whilst simultaneously maintaining tumour exposure and therapeutic efficacy (Minotti et al., 2004). Unfortunately, cardiotoxicity with these agents is still not prevented and further clinical strategies are required for management of the cardiac effects. Therefore, although cumulative dose is inherently related to the risk of cardiovascular toxicity, several other contributory factors are now known to be involved, including dosing rate and schedule, patient age, female gender, hypertension, previous cardiovascular disease, mediastinal radiotherapy and genetic predisposition (Brana and Tabernero, 2010, Lipshultz et al., 2013, Lotrionte et al., 2013, Cappetta et al., 2018). Despite exacting requisite caution when administering anthracyclines to these specific patients, the nature of chronic cardiovascular toxicity is such that myocardial dysfunction can present insidiously, many years after completion of therapy, again adding to the challenge of cardiotoxicity management. Consequently, in addition to identification and confirmation of risk factors, strategies for clinical management and potentially mitigation of anthracycline-induced cardiotoxicity are a high priority.

1.3.3 *Kinetics of cellular uptake of doxorubicin in cardiomyocyte and reflection of the clinical situation in vitro*

Even within with first hour of doxorubicin exposure, signs of intracellular oxidation and increases in reactive oxygen species are detectable within cardiomyocytes (Sarvazyan, 1996, Ludke et al., 2017). The mitochondria are the principal site of reactive oxygen species generation, with rapid doxorubicin accumulation observed both within these mitochondria and also the cardiomyocyte nuclei (Sarvazyan, 1996). The high nucleic accumulation of doxorubicin coupled with the ROS, induces apoptosis and creates important ultrastructural changes within cardiomyocytes. Endomyocardial biopsies sampled from patients receiving doxorubicin chemotherapy demonstrated early increases in mitochondrial and tubular size, whilst changes within the nuclei were also detected after only four hours of doxorubicin exposure (Arola et al., 2000, Unverferth et al., 1983, Unverferth et al., 1981). Cardiomyocytes are susceptible to oxidative damage, with limited anti-oxidative mechanisms, relative to other tissues (i.e. hepatic), to counteract the high levels of intracellular ROS formation (Hanf et al., 2019, Simůnek et al., 2009). In addition to the nuclear changes, mitochondrial and sarcoplasmic reticular swelling is observed, seemingly a precursor to anthracycline-induced cellular injury and cardiomyocyte apoptosis, whilst the surviving cardiomyocytes exhibited hypertrophy (Buja et al., 1973, Billingham et al., 1978, Chen et al., 2000). Furthermore, these mitochondrial and sarcoplasmic reticular manifestations may even explain the observed disturbances in intracellular calcium homeostasis. Consequently, reduced energy production caused by cellular membrane injury and increased oxidative stress influencing ATP-dependent calcium exchange, contribute to doxorubicin-mediated cardiomyocyte necrosis and culminate in a loss of myocardial function (Octavia et al., 2012, Zhang et al., 2009). Therefore, protection of the mitochondria and cardiomyocytes from ROS represent important anthracycline-induced cardiotoxicity therapeutic targets to be explored. Whilst explanations for anthracycline-induced cardiotoxicity mechanisms emphasise cardiomyocyte responses to doxorubicin exposure, future research should also consider the other myocardial constituents (i.e. fibroblasts, endothelial cells, smooth muscle cells), which are essential to cardiomyocyte homeostasis.

In susceptible individuals, anthracycline exposure induces cardiac myocyte and progenitor cell apoptosis. In most cases, this loss of cardiac myocytes is well compensated by increase in cell volume and there is no measurable reduction myocardial mass or function (Olivetti et al.,

1991). However, as part of the normal aging process, over 50 million cardiac myocytes are lost each year, so over time, particularly when associated with cardiovascular stress such as the development of hypertension, cardiac decompensation and clinical heart failure can occur. In older cancer patients, cardiac reserve is already reduced at the point of anthracycline exposure and therefore heart failure is more common even at lower anthracycline doses (Olivetti et al., 1991, Swain et al., 2003, Strait and Lakatta, 2012, Findlay et al., 2019).

Chronic anthracycline-induced cardiotoxicity is characterised by the clinical presentation of cardiac dysfunction beyond one year following chemotherapy. Therefore, during this latent period from initial doxorubicin exposure until clinical cardiotoxicity onset, there remains uncertainty surrounding the cardiomyocyte responses and adaptations.

1.3.4 *Histological changes in cardiac tissue associated with anthracycline treatment*

Histological analysis of these myocardial changes has continued to deepen our understanding and proposed solutions to the dilemma of chronic anthracycline cardiotoxicity. Biopsies taken from cancer survivors have demonstrated sequential myofibrillar loss and myocytolysis, together with dilatation of the sarcoplasmic reticulum (regulator of calcium ion concentration within cardiac muscle) and degenerative mitochondrial changes (Berry and Jorden, 2005). Histological studies of cardiac tissues from patients exhibiting chronic toxicity demonstrate myocardial necrosis, without inflammatory infiltrates, and in the latter stages diffuse interstitial fibrosis in conjunction with a dilated cardiomyopathy (Cascales et al., 2012). This contrasts with symptomatic acute anthracycline cardiotoxicity, which displays an inflammatory process and phenotype. This is indicative of cardiotoxicity presenting symptomatically acutely within hours/days and chronically within months/years as being different processes, with different immunological involvements. Cardiotoxicity presenting chronically thereby involving myocardial loss and compensatory processes, with progressive development from asymptomatic to symptomatic, rather than triggering a reactionary immune response. Consequently, not only do these cardiac microscopy changes inform cardiotoxicity presentation, they also provide an essential insight into the mechanisms of anthracycline cardiotoxicity and reveal key targets for preventative management strategies.

1.4 Molecular mechanisms of anthracycline toxicity against cardiac cells

1.4.1 Induction of DNA damage and inhibition of DNA synthesis

The mechanisms of anthracycline-induced cardiomyopathy are both complex and multifactorial, involving several potential processes. Anthracyclines, in addition to DNA intercalation, achieve their anti-neoplastic activity through interacting with topoisomerase II-α enzyme, forming topoisomerase-anthracycline-DNA complexes, DNA strand breaks and subsequent inhibition of DNA replication which leads to DNA damage and ultimately tumour cell death. Although this is successful in targeting the cancer, it also affects cardiac myocytes and induces cardiotoxic adverse effects (Negri et al., 1995, Barry et al., 2007). It is accepted that an initial effect of anthracycline treatment will be induction of cell death in susceptible cells within the cardiac myocardium, supported by the observation that high-dose anthracycline exposure causes nuclear and cellular morphological features consistent with apoptosis and necrosis in cardiomyocytes (Unverferth et al., 1983, Arola et al., 2000). However, the extent of these mechanisms in a largely quiescent cellular population and the relationship of this initial exposure to late-stage cardiac failure remains unclear.

Alternatively, whilst the therapeutic action of anthracyclines involves disruption of DNA-topoisomerase II-α complexes, it has been hypothesised that a second topoisomerase enzyme, II-β, expressed in cardiomyocytes may play an additional role in anthracycline-mediated cardiotoxicity (Zhang et al., 2012, Hahn et al., 2014). Deletion of the Top2-β gene, encoding for topoisomerase II-β in mouse models, promotes cardiotoxicity protection against acute anthracycline exposure, with a 70% reduction in cardiomyocyte cell death compared to those without the deletion. Furthermore, anthracycline treated Top2-β-knockout mice avoid cardiac injury with preservation of normal cardiac function, compared to the mild reduction in ejection fraction (53-43%) observed in the Top2-β-present group (Zhang et al., 2012, Hahn et al., 2014, Zhang et al., 2016).

1.4.2 Interruption of mitochondrial bioenergetics and function

The mitochondria, highly concentrated within cardiac tissue, are essential for oxidative phosphorylation and adenosine triphosphate (ATP) production (Lemieux and Hoppel, 2009). In addition to involvement of DNA damage mechanisms, anthracycline toxicity has principally been associated with the synthesis of oxygen free radicals, which are not only highly toxic to proteins and cellular lipids, but also cause mitochondrial failure and can themselves induce

DNA damage. With a high density of mitochondria, cardiac tissues are distinctly susceptible to any alterations in molecular signalling caused by the oxygen free radical damage together with suppression of cardiac mitochondrial metabolism, resulting in cardiac cellular dysfunction and myocyte death. Furthermore, reactive oxygen species (ROS) and active metabolites generated from anthracycline metabolism, inhibit the essential uptake of calcium ions through the progressive impairment of sarcoplasmic reticulum function. Disruption to the regulatory calcium-handling mechanisms through apoptosis and sarcoplasmic reticulum damage, limit cardiomyocyte contractility and contributes to the deterioration in ventricular function (Octavia et al., 2012). These mechanisms correlate strongly with both the histological and cardiac imaging findings, producing progressive cardiac dysfunction through cardiomyocyte necrosis and culminating in patients presenting to the clinic with signs of cardiac failure (Lipshultz et al., 2005, Carvalho et al., 2014).

To further establish the oxygen free radical hypothesis, there has been *in vivo* research investigating the potential of redox inhibitors and ROS scavengers to limit anthracyclines cardiotoxicity. Despite their administration, these studies failed to demonstrate any statistically significant benefit, which supports the argument for a multifactorial basis of anthracycline-induced cardiotoxicity (Pointon et al., 2010). The significance of the mitochondria-cardiotoxicity relationship is again emphasised by anthracyclines binding to the mitochondrial membrane protein cardiolipin, which increases mitochondrial anthracycline accumulation, leading to mitochondrial swelling and functional impairment (Wallace, 2003).

The pathways responsible for oxidative metabolism, which are stimulated by anthracycline therapy, are also closely linked with iron homeostasis to which anthracyclines have a high affinity. The adherence of anthracycline-iron complexes to the inner mitochondrial membrane promotes the synthesis of additional free radicals and toxic metabolites, these are accompanied by progressively high levels of intra-mitochondrial anthracycline accumulation, potentiating cardiac toxicity (Octavia et al., 2012, Gammella et al., 2014). The iron free radical hypothesis has been supported through the analysis of physiologically high iron states and studies of hereditary haemochromatosis. Both anthracycline exposure and patients with hereditary haemochromatosis, exhibit elevated intra-cardiac iron concentrations, which is consistent with a recent meta-analysis advocating the use of dexrazoxane, an iron chelator, especially if high doses of anthracyclines are anticipated (Cascales et al., 2012, Gammella et al., 2014, de Baat et al., 2022). In response to these findings, the use of dexrazoxane is

recommended within international clinical guidelines for the reduction of anthracycline-induced cardiac failure, however, concerns remain as to whether dexrazoxane also reduces anti-tumour efficacy, which until now has restricted its clinical availability (Macedo et al., 2019, Lyon et al., 2022). Despite these crucial therapeutic developments, clinical-based evaluation of stronger, more selective iron chelators for the prevention of ROS-mediated myocyte damage has demonstrated variable benefit, and therefore challenges the iron free radical and ROS hypothesis as a major mechanism of anthracycline-induced cardiotoxicity, and questions iron chelation, as opposed to another mechanism of action for dexrazoxane in this situation (Martin et al., 2009, Simůnek et al., 2009).

1.4.3 Induction of cellular senescence

From early adolescence, mature cardiomyocytes lose their early proliferative capacity and are therefore regarded as terminally differentiated cells (Bergmann et al., 2015). Even transient, low dose exposure to doxorubicin induces oxidative stress within these matured cardiomyocytes, with inflammation and mitochondrial DNA damage triggering apoptosis (Chularojmontri et al., 2013). The resulting mitochondrial damage and accelerated cardiomyocyte cellular losses culminates in progressive myocardial dysfunction consistent with a dilated cardiomyopathy (Mitry et al., 2020). Furthermore, expression of cellular senescence biomarkers (e.g. β-galactosidase, p16INK4a, and p53) are detectable within doxorubicin-treated cardiomyocytes, producing physiological effects such as progressive metabolic dysfunction and inhibition of cellular repair mechanisms, consistent with an accelerated ageing process of the cardiomyocytes (Maejima et al., 2008, Mitry et al., 2020). The inability of the cardiomyocyte repair and replacement mechanisms to counteract the accelerated cellular losses induced by anthracyclines leads to impaired cardiac contractility and an increased risk of cardiac arrhythmias. These senescence mechanisms may even be capable of impairing cardiac fibroblast activation and myocardial recovery, as non-cardiomyocytes (i.e. fibroblasts, endothelial cells and vascular smooth muscle cells) are implicated (Chen et al., 2021). The permanent cardiomyocyte changes, impaired compensatory responses and an accelerating ageing profile of cardiomyocytes may therefore explain the delayed or late-onset effects of anthracycline-induced cardiotoxicity (Mitry et al., 2020). Further research studies are required to evaluate the dose and time-dependent induction of myocardial senescence in response to doxorubicin.

In addition to impaired mitochondrial function, premature cardiomyocyte senescence may also play a crucial role in the development of late-onset cardiotoxicity (Mitry et al., 2020). The cardiac progenitor cells (CPC) located within the myocardium could therefore provide an essential protective function within these injury-repair mechanisms, regenerating cardiomyocytes damaged by anthracycline exposure. This could explain why certain patients develop late-onset cardiotoxicity, with significant damage to these CPCs rendering patients vulnerable to cardiac dysfunction, especially in the context of repeated anthracycline doses. Furthermore, the study of CPCs could provide the knowledge necessary to explain the clinical differences in anthracycline-induced cardiotoxicity between adult and paediatric patients, with increased cardiomyocyte sensitivity and CPC activity demonstrated in younger patients (Zhang et al., 2009, Witman and Sahara, 2018).

1.4.4 Cardiomyocyte structure and function

The adult mammalian heart is composed of several cell types including smooth muscle cells, fibroblasts, endothelial cells and cardiomyocytes. The presence of excitatory and conductive muscle fibres is essential for controlling cardiac rhythmicity, conducting action potentials efficiently between cardiac muscle cells to stimulate cardiac contraction generated by the fast sodium and slow calcium ion channels. Cellular influx and efflux of calcium regulates the force and speed of myocardial contraction, with this mechanism of ion propagation known as excitation-contraction coupling. Myofibril units present in the atria and ventricles contain actin and myosin filaments, which slide past one another to produce muscle contractions. Cardiomyocytes are therefore integral to cardiac muscle contraction and are connected via gap junctions to facilitate conduction (Guyton and Hall, 2011).

Mammalian cardiomyocyte development and proliferation begins during the foetal stages of life and is maintained beyond the neonatal period. The rate of cardiomyocyte proliferation then substantially decreases, as the cardiomyocytes preferentially undergo physiological hypertrophy to maintain cardiac development. The transformation into terminally differentiated cells is signalled by an absence of mitosis and cytokinesis (Woodcock and Matkovich, 2005, Foglia and Poss, 2016). Following these developmental stages, the total cardiomyocyte numbers remain relatively constant and are consistent well into adult life. Although, cardiomyocytes are gradually lost through processes of apoptosis and minor cardiac injury, a low baseline cardiomyocyte turnover is sufficient to maintain cardiomyocyte numbers and retain cardiac structural integrity (Bergmann et al., 2015).

This understanding of cardiomyocyte development has been challenged in one study which examined the cellular growth mechanisms within the left ventricle (Mollova et al., 2013). Mollova et al. used stereological analysis to demonstrate that cardiomyocyte development may be driven by both hypertrophy and proliferation mechanisms, with mean cellular volume and human cardiomyocyte numbers actually peaking in late adolescence. This hypothesis suggests that children and young adults sustaining cardiac injury have the potential to regenerate areas of damaged myocardium, whilst cardiac injury in adults is considered irreversible due to the cessation of cardiomyocyte cell division during adolescence (Mollova et al., 2013). Although later research from Bergman et al. also confirms hypertrophic growth within the cardiac ventricles, cardiomyocyte numbers evaluated using myocyte nuclear-specific markers and C^{14} isotope analysis reveals that nucleic numbers remain static after the initial peri-natal development phase, with continuous cardiomyocyte turnover highest in the first decade of life, before declining to 0.8% per year at age 20 years and 0.3% by 70 years of age (Bergmann et al., 2009, Bergmann et al., 2015). These findings suggest that the majority of cardiomyocytes are not exchanged during the human adult lifespan, rather it is the endothelial and the mesenchymal cell numbers which exhibit the endogenous repair mechanisms, with cellular exchange continuing at much higher rates into early adulthood relative to cardiomyocytes. The metabolically active endothelial cells are essential to the vascular integrity of the heart, regulating vascular tone and angiogenesis, whilst the mesenchymal cells (i.e. fibroblasts, pericytes and smooth muscle cells), located within the cardiac interstitium, principally promote cardiac regeneration throughout the human lifespan. Both whole cell populations renew every few years during an adult lifetime, with endothelial cells exhibiting the highest rates of regeneration (Bergmann et al., 2015). These are significant findings in relation to cardiotoxicity, as they further our understanding of the mechanisms and the cellular responses to cardiac injury.

Under conditions of physiological stress and mild cardiac injury, cardiomyocytes have been observed to undergo hypertrophic growth responses, with functional myocytes adequately compensating for any myocyte losses incurred, to maintain cardiac output (Woodcock and Matkovich, 2005). Although cardiomyocytes have demonstrated some regenerative capacity, in conditions causing significant myocyte apoptosis and necrosis, the loss of cardiomyocytes is largely irreversible. *In vitro* studies have demonstrated that cardiomyocyte repair can originate from cardiac stem cells and progenitor cells. However, when these cells incur damage, their

regeneration and repair mechanisms are inhibited. Instead, cellular renewal may be confined to the endothelial and mesenchymal cells, but rather than improving contractile ability this initiates fibroblast proliferation. In cases of significant cardiac injury, this fibroblast proliferation accelerates the type of cardiac fibrosis frequently observed in ageing hearts and leads to permanent myocardial scarring. This ultimately limits the contractile potential of the heart and leads to cardiac failure (Bergmann et al., 2009, Guo and Pu, 2020).

1.4.5 Non-cardiomyocyte cells within the heart and the effects of anthracyclines

Cardiac fibroblasts are derived from mesenchymal origin, forming part of the extracellular matrix within the dynamic cardiac structure. In addition to providing structural support for the cardiomyocytes, fibroblasts maintain cardiac tissue integrity through the production of extracellular matrix proteins and growth factors to counterbalance the tissue degradation incurred by ageing and cardiac injury. Fibroblasts are highly sensitive to pathological stimuli (e.g. myocardial infarction, heart failure, cardiac hypertrophy), initiating proliferative responses and tissue remodelling pathways to maintain optimal cardiac output (Camelliti et al., 2005). In response to changes in cardiac physiology, cardiac fibroblasts are activated and converted into myofibroblasts, which in turn deposit connective tissue, including collagen and glycosaminoglycans, within normal cardiac tissue to promote cardiac remodelling. Inflammatory cells such as macrophages and monocytes are also triggered in response to cardiac damage, stimulating fibroblast recruitment and promoting growth factors to support cellular repair (de Boer et al., 2019). The subsequent cardiac fibroblast proliferation can be described as either reparative or reactive fibrosis, depending upon the primary aetiology and the accompanying histological features. Both fibrosis types result in thickened and scarred tissue, which creates increased myocardial stiffness, impairing both systolic and diastolic cardiac functions, whilst the scarred regions have an increased potential to initiate fatal cardiac arrhythmias (Weber, 1989).

The transition of cardiac magnetic resonance imaging (CMR) into cardio-oncology has enhanced our understanding of cardiac remodelling in response to anthracycline chemotherapy. In addition to evaluating cardiac structure and function, CMR has the capacity to characterise myocardial tissue in patients receiving anthracyclines. The development of myocardial fibrosis has been shown to manifest later within the course of anthracycline-induced cardiotoxicity and has the potential to become a marker for future myocardial dysfunction (Bernaba et al., 2010, Farhad et al., 2016). Measurements of extra-cellular volume

and T_1-weighted mapping have supported the assessment of myocardial fibrosis in patients treated with anthracycline chemotherapy. However, results from recent animal studies have shown some inconsistencies when compared with human studies (Cove-Smith et al., 2014, Muehlberg et al., 2018, Galán-Arriola et al., 2019). Further research is required in this field and may be best supported with serum biomarker studies for interstitial fibrosis (e.g. metalloproteinases (MMP), procollagen peptides and tissue inhibitors (TIMP)), to identify early cardiac remodelling and best predict anthracycline-induced cardiotoxicity.

Damage to the endothelial cells caused by anthracycline chemotherapy can accelerate the development of cardiac failure, as these cells are responsible for promoting coronary blood vessel formation and growth. They support cardiomyocyte homeostasis through helping the transfer of nutrients directly to the cells, whilst also forming as a protective layer which surrounds the cardiomyocytes. Endothelial cells appear to be susceptible to the reactive oxygen species (ROS) produced by anthracycline chemotherapy, which disrupts these homeostatic mechanisms and overwhelms the antioxidant capacity of the endothelial cells (Luu et al., 2018). Progressive damage to the endothelial cells by anthracyclines not only renders the cardiomyocytes vulnerable to direct damage from the chemotherapy by increasing endothelial permeability, but also appears elevates the risk of atherosclerosis and future cardiac ischaemia as observed in long-term paediatric cancer survivors (Chow et al., 2006, Lipshultz et al., 2013). The endothelial dysregulation caused by anthracycline exposure appears to significantly worsen the degree of cardiomyocyte damage, therefore future therapies targeting the preservation or enhancing endothelial function should be evaluated for the prevention of anthracycline-induced cardiotoxicity (Luu et al., 2018).

1.4.6 Role of cardiac progenitor cells

The study of cardiac progenitor cells (CPCs) may enhance our understanding of why certain patients treated with anthracycline chemotherapy display late-onset cardiotoxicity. CPCs are divided into two types: embryonic/developmental CPCs and adult CPCs. In addition to supporting growth and development of the myocardium through the formation of cardiomyocytes, smooth muscle and endothelial cells, CPCs also help maintain cardiac tissue homeostasis and promote myocardial regeneration. *In vitro* studies have demonstrated that anthracycline chemotherapy causes a combination of cardiomyocyte death and inhibits the proliferation of CPCs (De Angelis et al., 2016, Witman and Sahara, 2018). Neurohormonal activation, sympathetic stimulation and mechanical adaptations are the principal adaptions in

preserving cardiac function after cardiac injury. However, if these compensatory signalling pathways continue to be unregulated, they may lead to cardiac failure in the long-term (Mann and Bristow, 2005). The CPCs remain essential to preserving and promoting cardiac function, through the structural and functional recovery of cardiomyocytes and surrounding cells. In addition to inhibiting CPC proliferation, anthracyclines deplete absolute cardiac stem cell numbers through direct toxicity, whilst also accelerating cellular senescence as shown by age-matched patient controls. Following death and damage to the CPCs, the impaired turnover of cardiomyocyte and accompanying cardiac cells renders the myocardium vulnerable to late-onset cardiomyopathy (Piegari et al., 2013, De Angelis et al., 2016). Therefore, when patients with sub-clinical cardiotoxicity are exposed to cardiac stressors (e.g. future myocardial injury, high-output cardiac states), they are at increased risk of developing overtly symptomatic cardiac failure, which conveys substantial mortality rates, reducing cancer overall survival rates.

1.5 The role of the cardio-oncologist

Cardio-oncology is a rapidly emerging cardiology sub-speciality focussing upon the cardiac care of cancer patients. The role of the cardio-oncologist is to manage both pre-existing cardiac conditions and new cardiac issues identified during cancer therapy, to ensure patients successfully receive their cancer treatment and maximise their chance of improved overall survival. Certain chemotherapy treatments are known to cause adverse cardiac effects, ranging from acute and short-term post-infusion toxicities, through to chronic and long-term cardiovascular effects, often presenting decades after the completion of cancer treatment.

Consequently, the successes associated with improvements in overall cancer prognosis and treatment are counterbalanced by detrimental life-threatening effects upon the cardiovascular system, culminating in higher rates of cardiovascular morbidity and mortality. Studies are therefore required to better understand these mechanisms, identify risk factors, and optimise treatment regimens to mitigate these adverse effects and improve the quality of life of cancer patients.

1.6 ***Management of cardiotoxicity caused by oncology therapeutics***

Cardiotoxicity preventative and treatment strategies have focussed upon understanding the mechanisms of anthracycline chemotherapy, seeking to target specific points within the therapeutic pathway to prevent adverse cardiovascular effects. The caveat to this approach is

that pharmacological agents targeted at mitigating cardiotoxicity may impair the intended therapeutic action of the chemotherapy, exposing patients to the risks of incomplete tumour eradication or cancer recurrence. Whilst the management of acute cardiotoxicity seems to be manageable given its short-term reversibility, the development of chronic cardiovascular toxicity is significantly more challenging, therefore requiring innovative approaches to prevent cardiovascular events and maintain the strong therapeutic efficacy of cancer therapy.

Understanding the mechanisms of cardiotoxicity and identifying strategies for management are pivotal within cardio-oncology, given the significant impact cardiotoxicity has upon the clinical outcomes for patients with malignancy and their cancer treatment. The demonstration that anthracycline chemotherapy is associated with a high risk of long-term progressive cardiotoxicity emphasises the importance of implementing effective management strategies to treat chemotherapy-induced cardiotoxicity. Therefore, the key focus of cardiotoxicity research must now evolve towards prevention, with studies now illustrating that intervening after an observed rise in cardiac biomarkers or echocardiographic changes is potentially too late to avoid the consequences of long-term cardiotoxicity. Treatment and prevention strategies have been targeting different stages of the therapeutic pathway, in addition to analysing potential cardiac biomarkers for prevention of these adverse cardiovascular effects.

1.6.1 *Administration of inhibitors of the beta-adrenoceptor; beta-blockers*

Inhibitors of the beta adrenoceptor (β-blockers) such as carvedilol are used for the treatment of heart failure. Carvedilol, which antagonises beta (β1, β2) and alpha (α1) adrenergic receptors, is unique amongst β-blockers as it also possesses antioxidant properties (Book, 2002). In contrast to carvedilol, selective β-blockers, such as atenolol, target β1-adrenoceptors to decrease the rate and force of myocardial contraction, but do not significantly affect α1-adrenoceptors (Weber, 2005). Blockade of α1-adrenoceptors, as is the case with carvedilol, drives vasodilation, associated with reduced blood pressure and as such carvedilol in theory may be more effective at treating heart failure than selective β-blockers (Poole-Wilson et al., 2003, Remme, 2010). With respect to reduction of anthracycline-induced cardiotoxicity, in contrast to the selective beta-blocker atenolol, carvedilol has been shown to decrease production of free radicals and retard death of rat cardiomyocytes caused by anthracyclines *in vitro* (Spallarossa et al., 2004). This suggests that the antioxidant properties of carvedilol are beneficial and that this may protect the heart from anthracycline-induced cardiotoxicity, a strategy that has been employed in clinical trials (Kheiri et al., 2018). Encouraging results have

been demonstrated with prophylactic administration of carvedilol, with these drugs maintaining both systolic and diastolic functions of the left ventricle in patients undergoing anthracycline therapy (Kalay et al., 2006, Kheiri et al., 2018).

1.6.2 *Disruption of angiotensin signalling as a strategy for mitigation of anthracycline-induced cardiotoxicity*

The myocyte death and myocardial dysfunction observed with anthracycline cardiotoxicity are also frequently observed in common cardiac conditions such as congestive cardiac failure and myocardial infarction, with secondary prevention strategies aimed at both preventing progression of left ventricular impairment, adverse remodelling and potential restoration of cardiac function. It is well established that clinical management of hypertension and heart failure is centred around optimisation of cardiac haemodynamics, particularly through the renin-angiotensin-aldosterone system (RAAS) axis.

The RAAS is essential for regulation of cardiovascular, renal and vascular physiology. Angiotensin is a group of plasma proteins derived from angiotensinogen, an alpha-2 globulin released into the circulation by the liver. Through a series of enzymatic cleavages involving renin and angiotensin converting enzyme, the physiologically active octapeptide angiotensin II is synthesised. Using a complex receptor signalling pathway, angiotensin II exerts its effects upon the cardiovascular, renal, adrenal and central nervous systems.

The principal systemic effects of angiotensin II include arteriolar vasoconstriction and regulation of plasma volume through sodium and water reabsorption, activated through systemic volume depletion. Through its influence upon cardiac output and arterial pressure, the RAAS is essential for preserving end-organ perfusion and function. Whilst these adaptive responses are vital for maintaining blood pressure and correcting physiological volume depletion, persistent changes in cardiac preload and afterload can influence long-term cardiac physiology. Increases to ventricular afterload, result in reduced left ventricular emptying and impaired myocardial relaxation. As a consequence, angiotensin II stimulates an increase in left ventricular end-diastolic pressure and subsequent cardiac hypertrophy (Ruzicka and Leenen, 1999). These pathophysiological effects may then play a role in the development of important cardiac pathology such as hypertension, heart failure and vascular disease.

Further to the vascular effects, angiotensin II also has a direct influence upon myocardial tissue through alteration of myocardial contractile function and further upregulation of the cardiac

hypertrophic response. Cardiac myocytes demonstrate a high affinity for angiotensin II, with cell-surface activation of the angiotensin 1 (AT1) receptor (AT1R), a sub-class of the angiotensin II receptor, located on ventricular fibroblasts and adult cardiomyocytes. Activated by angiotensin II through reversible binding, AT1Rs closely regulate cardiovascular and renal physiology, producing cardiac effects of pressure regulation, cellular growth and remodelling, with additional receptor sites located in target organs including the adrenals, brain, liver and lungs (Mehta and Griendling, 2007). Furthermore, the AT1R has two subtypes, type A (AT1*A*) and type-B (AT1*B*) receptors, with *type-A* receptors essential for cardiomyocyte growth and blood pressure regulation, whilst *type-B* receptors optimise vascular tone in the absence of AT1*A* stimulation (De Mello and Danser, 2000).

Angiotensin II receptors are widely expressed within the atrial and ventricular myocardium, enabling them to enhance the inotropic and chronotropic properties of the heart to maintain stroke volume (Allen et al., 2000). Whilst the haemodynamic effects induced by volume and pressure overload appear to be the principal stimuli for cardiac hypertrophic transformation, angiotensin II binds to specific membrane-bound receptors to initiate multiple intracellular transduction pathways, resulting in direct growth-promoting effects and pathological cardiac remodelling (Ruzicka and Leenen, 1999, Allen et al., 2000). Therefore, through binding to the angiotensin II receptors located on ventricular cardiomyocytes, circulating angiotensin II establishes strong cardiovascular adaptive responses including the mediation of cellular growth, optimisation of cardiac contractility and initiating cardioprotective mechanisms.

Angiotensin II not only drives blood pressure but can influence left ventricular mass and left ventricular remodelling through mechanisms involving collagen expression, perturbation of cardiac haemodynamic loading, and regulation of the cardiac hypertrophic response (Widdop et al., 2003, López-Sendón et al., 2004). The clinical pathophysiology of anthracycline cardiotoxicity is closely associated with congestive cardiac failure, with the latter stages of toxicity displaying a progressive reduction in LVEF and echocardiographic appearances consistent with dilated cardiomyopathy. Therefore, hypothesising that angiotensin converting enzyme inhibitors (ACEi) can have the same beneficial effect in patients with anthracycline-induced cardiomyopathy as in conventional heart failure seems highly plausible.

1.6.2.1 Angiotensin converting enzyme inhibitors (ACEi) for mitigation of anthracycline-induced cardiotoxicity

ACEi propagate a natriuretic effect through reduction in angiotensin II levels, whilst activation of angiotensin II receptors located within cardiac myocytes, adapt myocardial contractile function and upregulate the cardiac hypertrophic response. Significant benefits of ACEi have been demonstrated for management of left ventricular systolic dysfunction, through large-scale clinical trials (CONSENSUS, 1987, SOLVD, 1991, Jong et al., 2003). Administration of the ACEi enalapril was shown to reduce mortality by 40% at 6 months and 31% at 12 months in the CONSENSUS trial (CONSENSUS, 1987). This outcome, alongside that of other studies, supports a mechanism for the effects of ACEi upon survival in heart failure aligned to hormone suppression rather than haemodynamic improvements per se (Garg and Yusuf, 1995).

The survival benefits of ACEi in heart failure, in combination with the fact that anthracycline-induced cardiotoxicity is associated with progressive heart failure, led to the use of ACEi as a potential clinical strategy for patients with anthracycline-induced cardiotoxicity (Cardinale et al., 2006). This hypothesis was shown to have validity in subsequent clinical studies, as ACEi administration to cancer patients who had received high-dose anthracycline chemotherapy led to a slowed progression of left ventricular dysfunction (Cardinale et al., 2006). Within this clinical trial, Cardinale et al. randomised patients with evidence of cardiomyocyte injury (elevated serum troponin I) following high-dose chemotherapy, to receive an ACEi (enalapril) or not, with reduction of >10% in ejection fraction as the trial's primary end point. Their study found that patients receiving prophylactic enalapril were prevented from developing cardiotoxicity and adverse cardiovascular events (Cardinale et al., 2006). This study advanced the work ten years earlier from Jansen et al., who initially learned that patients with evidence of symptomatic cardiotoxicity, defined as symptomatic left ventricular impairment after epirubicin chemotherapy, experienced a normalisation of left ventricular ejection fraction (LVEF) within 3 months of commencing an ACEi, which was sustained throughout mid-term follow-up (Jensen et al., 1996). These cardioprotective strategies utilising ACEi have been extended into paediatric patients, with results from Gupta and colleagues concluding that paediatric patients receiving early ACEi therapy were more likely to have a preservation of LVEF and maintain normal levels of cardiac biomarkers (troponin I, pro-BNP and CK-MB) upon exposure to cardiotoxic doses of anthracyclines (Gupta et al., 2018). However, a small-population study from Lipshultz et al. evaluating the long-term effects of enalapril for left

ventricular dysfunction in paediatric patients previously treated with doxorubicin (at least one year post-completion) similarly recognised an improvement to left ventricular structure and function, however this was only transient, with echocardiographic parameters deteriorating during extended follow-up between six and ten years (Lipshultz et al., 2002).

There are several proposed hypotheses to explain the cardioprotective effects of ACEi, ranging from haemodynamic effects as a consequence of reduced afterload, decreased systolic ventricular wall stress and lowering of plasma catecholamine levels (Gavras et al., 1978, Dzau et al., 1980, Janbabai et al., 2017), mitigating oxidative stress and interstitial fibrosis (Abd El-Aziz et al., 2001, Yusoff et al., 2017), and preservation of mitochondrial function and cardiac ATP production (Hiona et al., 2011, Taskin et al., 2016). Although the contribution of these mechanisms to mitigation of anthracycline-induced cardiotoxicity is as yet unconfirmed, ultimately their effects are a direct result of an improvement to cardiac output due to optimisation of haemodynamic forces and reduced adverse ventricular remodelling (López-Sendón et al., 2004).

1.6.2.2 PRevention Of Anthracycline Cardiovascular Toxicity (PROACT) Clinical Trial

Several clinical trials have demonstrated the benefit of ACEi administration to patients presenting with symptomatic anthracycline-induced cardiotoxicity, with these data implying earlier administration of ACEi would have greater effects (Cardinale et al., 2006, Cardinale et al., 2010). Meta-analyses acknowledge the limited evidence surrounding the efficacy of ACEi in the prevention of cardiotoxicity (Kalam and Marwick, 2013, Conway et al., 2015). Although a statistically significant treatment benefit with ACEi for existing cardiotoxicity has been observed, one significant limitation from these cardiotoxicity prevention trials is the administration of sub-optimal doses when compared to cardiology guideline recommendations and clinical trial target doses (Ponikowski et al., 2016, Ibanez et al., 2018). In this context, PROACT, an observational, prospective, phase III, randomised controlled clinical trial involving The Newcastle Hospitals NHS Foundation Trust, designed to evaluate the effectiveness of ACEi for the prevention of cardiotoxicity in breast cancer and non-Hodgkin's lymphoma patients (NCT03265574). In contrast to other studies involving administration of ACEi either following conclusion of chemotherapy or in symptomatic cancer patients, PROACT involves prophylactic administration of an ACEi (enalapril) prior to commencement of anthracycline chemotherapy and continuation throughout the treatment period. To assess the effectiveness of this approach, blood samples are collected after each chemotherapy cycle to

measure cardiac troponin and other cardiac biomarkers. Patients are also subject to an echocardiogram at baseline and after treatment, to detect myocardial damage. The outcomes of this trial will improve understanding of the association between ACEi and anthracycline-induced cardiotoxicity, therefore supporting the objectives of this project focused on elucidation of the mechanisms of anthracycline-induced cardiotoxicity.

The PROACT trial intends to evaluate the clinical effectiveness of optimal ACEi therapy, ensuring patients receive the guideline-driven maximum tolerated dose, to determine the full effect cardiotoxicity prevention with ACEi. The interesting concept from these earlier trials is the spectrum of clinical responses to the ACE inhibitor therapy, although the majority of patients experienced a clinical improvement or complete reversal of cardiotoxicity, there were patients who continued to progress towards end-stage cardiac failure despite heart failure therapy. Cardio-oncology guidelines have accordingly transitioned towards preventative strategies for cardiotoxicity, advocating a proactive approach to cardiovascular risk assessment and minimising this risk through primary prevention strategies. However, the reasons for why certain patients experience cardiotoxicity from anthracycline chemotherapy whilst others benefit from treatment, could go well beyond the optimisation of ACEi therapy and be in fact be realised within the underlying mechanisms of the drugs.

1.6.2.3 Angiotensin receptor blockers (ARBs) for mitigation of anthracycline-induced cardiotoxicity

Angiotensin II receptors are located on cardiac myocytes, and their activation they alter myocardial contractile function and upregulates the cardiac hypertrophic response. Angiotensin receptors blockers (ARBs) are recognised within cardiovascular guidelines for the treatment of cardiac failure, as an alternative therapy for patients intolerant of ACEi, after they demonstrated a reduction in cardiovascular morbidity and mortality in patients with symptomatic heart failure (Granger et al., 2003, Ponikowski et al., 2016). ARBs also exert their influence upon the RAAS system, however in contrast to ACEi, ARBs compete with angiotensin II to bind to the angiotensin II receptor type-1 (AT1R). ARBs directly influence the circulating levels of angiotensin II, therefore stimulating vasodilatation to reduce vascular resistance and cardiac afterload, whilst simultaneously reducing the growth-stimulating effects of angiotensin II upon the heart. In addition, studies have detailed the direct cardiac effects of angiotensin II on myocardial hypertrophy, in addition to positive inotropic responses (Baker et al., 1992, Sadoshima and Izumo, 1993). These effects were confirmed to be mediated by the

AT1R located within the cardiomyocytes and through selective blockade of the AT1R with ARBs. Furthermore, these effects were also associated with suppression of genetic markers of the hypertrophic response (Sadoshima et al., 1993).

Exploration of the ARB mechanisms has revealed important links between angiotensin II and anthracycline therapy to explain the effectiveness of cardioprotection therapy. Similar to the case with ACEi, ARBs have now also been proposed for the treatment and prevention of anthracycline-induced cardiotoxicity, with animal and patient studies both displaying their benefit (Soga et al., 2006, Gulati et al., 2016). Evaluation of cardiovascular changes in doxorubicin-treated AT1R knockout mice and wild type mice concluded that blocking the AT1R provided a cardioprotective effect, with preservation of cardiac function compared to the wild type mice group which experienced increased cardiac myofibril loss, greater cardiomyocyte apoptosis and a significant reduction in left ventricular contractility (Toko et al., 2002). Cadeddu et al. progressed these findings by randomising patients with solid tumour cancers to receive telmisartan (an ARB) or placebo prior to anthracycline chemotherapy. They detected a protective effect due to angiotensin receptor blockade and a preservation of echocardiographic parameters at epirubicin doses greater than 200mg/m^2 (Cadeddu et al., 2010). With regards to the mechanism underpinning these beneficial effects, previous studies in our laboratory have demonstrated the induction of cardiomyocyte hypertrophy following exposure to anthracyclines, an effect that continues to occur at concentrations significantly lower than those inducing cardiomyocyte death (Rockley and Gill, 2017). Importantly, this hypertrophic response was shown to be similar to that observed following exposure of cardiomyocytes to pro-hypertrophic angiotensin II (Rockley and Gill, 2017). In support of this, selective blockade of AT1R, located on the surface of cardiomyocytes, has been shown to mitigate this anthracycline-induced hypertrophic response, linking the cardiac hypertrophic changes observed with angiotensin II and potentially explaining why ACEi may prevent anthracycline-induced cardiotoxicity (Jacobi et al., 1999, Rockley and Gill, 2017). In terms of the mechanistic basis of these responses, it is hypothesised that the induction of hypertrophy by anthracyclines is an adaptive response to the loss of cardiomyocyte structural integrity or the loss of cardiomyocytes as a consequence of direct cytotoxicity. At the cellular level, onset and progression of pathological cardiac hypertrophy is known to associate with alterations in mitochondrial function, morphology and dynamics (Vásquez-Trincado et al., 2016). In this context, associations between anthracycline and the mitochondria have been identified, with mitochondrial swelling and sarcoplasmic reticular dilatation have been observed

microscopically following anthracycline exposure (Mortensen et al., 1986). These studies collectively emphasise the significance of the AT1R and of mediating angiotensin II in preventing doxorubicin-induced cardiac dysfunction. Ultimately, elucidation of the mechanisms involved in these responses is central to the establishment of the precise mechanism linking the therapeutic efficacy of the ACEi and anthracyclines. If the adverse cardiovascular effects of anthracycline chemotherapy can be reduced or even prevented by the administration of ACEi or ARBs, then the protective effects are likely to be mediated (at least in part) through AT1R located on cardiomyocytes.

1.6.2.4 Genetic polymorphism of ACE and anthracycline-induced cardiotoxicity

A complexity for the management of anthracycline-induced cardiotoxicity is that patients with relatively low cumulative doses of anthracycline chemotherapy can develop chronic cardiotoxicity, whilst other patients with high cumulative doses experience no visible cardiovascular adverse effects (Swain et al., 2003). Given that the pathophysiology of anthracycline-induced cardiotoxicity is multi-factorial and manifests to different degrees and timescales in patients, a further dimension to appreciating the basis of these effects is determination of genetic predisposing factors. Such information would permit both stratification of patients at risk of developing late-stage anthracycline-induced cardiotoxicity and monitoring of asymptomatic patients for development of progressive heart failure. It is well reported that there is high variability in both the expression and activity of ACE in the population, associated with the presence of an intronic insertion/deletion (I/D) genetic polymorphism within the ACE gene, resulting in three genotypes: homozygous insertion (II), heterozygous insertion/deletion (ID), and homozygous deletion (DD) (Rigat et al., 1990). A defined association is observed between the DD genotype and left ventricular hypertrophy, with this being independent of confounding variables, such as blood pressure, heart rate, and body mass index (Schunkert et al., 1994). The DD genotype has also been shown to be a predictor of mortality in patients with idiopathic heart failure, with >20% difference in survival between DD and non-DD genotypes (Andersson and Sylvén, 1996). No studies have yet evaluated whether this genotype contributes to heart failure resulting from anthracycline-induced cardiotoxicity.

1.7 Thesis aims and objectives

The cardioprotective potential of therapies focused on disruption of angiotensin signalling has gained significant support in recent years, albeit by as yet unidentified or unconfirmed molecular mechanisms. The aim of this thesis is to evaluate the relationship between development of anthracycline cardiotoxicity and the angiotensin-signalling pathway, in order to explain why ACEi may reduce anthracycline induced cardiotoxicity and improve clinical management of this condition.

The overall objectives of this project are:

I. To establish and qualify *in vitro* cardiomyocyte models for evaluation of cellular responses and elucidation of molecular mechanisms of drug-induced toxicity

II. Evaluate the molecular relationship between anthracycline-induced cardiac cell toxicity and the angiotensin signalling pathway, using cardiomyocyte cell models *in vitro*

III. Assess relationship between ACE polymorphic genotype and development of anthracycline-induced cardiotoxicity, retrospectively in patients

IV. Characterise clinical relationships between anthracycline-induced cardiotoxicity, perturbation of angiotensin-signalling, and cardiovascular risk factors, with potential for translation into clinical patient care

Chapter 2. Materials and Methods

All laboratory reagents were obtained from Sigma-Aldrich Company Ltd (UK) unless otherwise stated. Plastic tissue culture materials were supplied by Sarstedt (UK).

2.1 Cell Lines and in vitro *maintenance*

The proliferating human cardiomyocyte cell line AC10 was utilised as the preclinical cardiotoxicity model within these studies, kindly donated by Dr Barbara Savoldo from Texas Children's Hospital, Texas, USA. This cell line derives from fusion of primary cultures of adult ventricular myocardial cells with SV40-transformed fibroblasts, therefore establishing an immortalised cardiomyocyte cell line (Davidson et al., 2005). These cells display a similar phenotype to adult ventricular cardiomyocytes with the presence of cardiac markers such a troponin-I, troponin-T and tropomysin, but are non-contractile.

Cells were maintained in Dulbecco's Modified Eagle medium (DMEM)/F12 medium supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin, grown as monolayers *in vitro* and incubated at 37°C in a constant humidified atmosphere of 5% CO_2. Cells were passaged when flasks were around 75-80% confluent by washing the monolayer with Hanks balanced salt solution (HBSS) and addition of 0.25% trypsin-EDTA, a pellet was then obtained by centrifugation at 400 xg for 5 minutes, with the cells re-suspended in fresh medium and either passaged into a new flask, or cell pellets were stored at -20 °C for molecular analysis.

2.1.1 Cell counting by haemocytometer

Cell number was determined using a haemocytometer where 10 µl of cell suspension was pipetted under a coverslip and the number of cells counted in five x 1mm^2 sections. Due to the dimensions of the haemocytometer the average value of cells (N) in the five sections equates to N x10^4 cells/mL.

2.2 Cryopreservation of cells

For cryostorage of cells, confluent AC10 cells were trypsinized and centrifuged at 400 xg for 5 minutes, the supernatant was removed, and the cells re-suspended in 1mL of fresh medium. Cells were transferred to a 2.0mL cryovial and 10% dimethyl sulfoxide (DMSO) was added. Cryovials were placed in a cryogenic Nunc freezing container, containing isopropanol, and

placed in a -80°C freezer. The following day cells were transferred to a liquid nitrogen storage dewer for long-term storage.

2.3 Cell proliferation and viability, evaluated by MTT metabolism assay

To establish *in vitro* growth responses and drug cytotoxicity in AC10 cells, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assays were conducted. The mitochondrial reductase enzymes (NAD(P)H-dependent oxidoreductase) within living cells reduces the MTT reagent to produce formazan crystals; the absorbance of which is measured at 550 nm using a spectrophotometer plate reader (Multiskan Go-Thermo Scientific®). The production of formazan crystals directly correlates to the mitochondrial activity and therefore is taken as a representation of cellular viability (Mosmann, 1983, Morgan, 1998). Cell viability is determined indirectly from cardiomyocyte cell membrane integrity, with a compromised cell membrane allowing dye uptake within the cell. The ratio of viable (absence of dye uptake) and non-viable (dye uptake) as determined by haemocytometer cell counting were calculated and expressed in terms of percentage viability.

2.3.1 Cell proliferation, assessed by MTT metabolism assay

For optimisation of the MTT assay for assessment of cellular viability and proliferative response, AC10 cells were seeded at densities ranging from 100-100 x 10^3 cells per well of a flat-bottomed 96-well cell culture plate, with one lane of the plate devoid of cells to act as a cell blank control. MTT assay analyses were conducted over a 6-day period (one 96-well plate per 24-hour time point), with day zero being the time of cell seeding. After each 24-hour incubation period at 37°C in 5% CO_2, the media was removed from all wells, the cells rinsed with HBSS to remove cellular debris and dead cells, 100 µL 1:10 dilution of MTT solution (5mg/mL, diluted in fresh culture medium) added to each of the 96 wells, and the plate re-incubated at 37°C for 4 hours to allow formation of the purple formazan crystals. After incubation, all cell media was carefully removed leaving only the insoluble formazan crystals. 175µL of dimethyl sulfoxide (DMSO) was added to each well and the crystals dissolved, and the absorbance determined at 550nm by spectrophotometry. Analysis of absorbance readings were conducted using Microsoft Excel; the average absorbance for each treatment group was determined from which the average of the blank lane was deducted, and average absorbances calculated. All experiments were completed in triplicate. Statistical analysis was performed using SPSS, with graphs constructed using either Microsoft Excel (Microsoft Corporation, 2018

or GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com).

Through optimisation of cells seeding densities, the exponential and plateau phases of growth were established. Future cell viability experiments were conducted using a standard cell seeding density of 2000 cells per well, with cells cultured for differing lengths of time prior to drug treatments, to best represent the exponential (3 days' culture time) and plateau (5 days) phases of cardiomyocyte growth.

2.3.2 Cell viability and drug cytotoxicity, determined by MTT metabolism assay

MTT assays were performed to evaluate the cytotoxicity of doxorubicin, establishing quantitatively the cellular viability of AC10 cardiomyocytes following exposure to different treatment doses. AC10 cells were seeded at 2000 cells per well in a 96-well plate and cultured in 200µL DMEM until entering either the exponential (3 days) or plateau (5 days) phase of growth, as determined by previous growth curves. One column remained cell-free, with media only, acting as a blank control for the experiments. Cells were then treated for 24 hours with serial dilutions of doxorubicin, ranging from $4x10^{-10}$ to $1x10^{-5}$ M, with each drug concentration replicated 8 times, therefore producing 8 intra-experimental replicates. The initial three lanes acted as experimental controls: media only, cells plus media, and a vehicle control (0.1% DMSO). After the required incubation period, the medium was removed, cells were gently rinsed with HBBS, and diluted MTT solution was added to each well. The procedure outlined in section 2.3.1 was adhered to for subsequent MTT assay analysis. Cell viability was expressed as a percentage relative to the vehicle control, with the mean and standard error of the mean (SEM) calculated. All studies were repeated in triplicate. The half maximal inhibitory concentration (IC_{50}) was calculated by combining the replicate values and using non-linear regression for curve fitting on GraphPad Prism. Statistical analysis for the IC_{50} values was completed using a one-way analysis of variance (ANOVA) test, and a post-hoc Dunnett's test. Comparisons between the doxorubicin concentrations were completed using Mann-Whitney test.

2.3.3 Trypan blue dye exclusion

Trypan blue exclusion analysis was also used to measure cell viability of AC10 cardiomyocytes treated with serial dilutions of doxorubicin, helping to distinguish between viable and non-viable cells following treatment. The assay is based upon the premise that viable cells maintain

an intact cell structure, remaining impermeable to trypan blue dye. Damaged cells however lose their plasma membrane integrity, absorbing the dye to stain the cytoplasm blue, and are therefore recorded as non-viable (i.e. cell membrane lysis equating to cell death). The ratio between the viable and non-viable cells was quantified using a haemocytometer, with the percentage cell viability of the cardiomyocytes in response to doxorubicin treatment subsequently calculated (Louis and Siegel, 2011).

AC10 cardiomyocytes were again seeded at 2000 cells per well in a 96-well plate, culturing in 200 µL DMEM until exponential or plateau growth phases. Serial dilutions of doxorubicin, ranging from 10µM to 39nM, was added to 8 vertical wells, representing one treatment dose and 8 replicates.

After 24 hours of treatment exposure, the media from each doxorubicin dose was then extracted and pipetted into a 15 mL falcon tube, as this would contain the majority of non-viable cells which had lost adherence to the plate during the 24-hour incubation period. The remaining cells were washed with HBSS, which was added to the falcon tube. The cardiomyocytes were trypsinised with 10µL pipetted into to each well to remove the adhered cells, and after a brief incubation period, they too were transferred to the falcon tube together with an equal volume of media to neutralise the trypsin. The combined cardiomyocytes for each treatment dose were centrifuged for 5 minutes at 100 xg to produce a cell pellet, with the supernatant extracted and discarded. 0.4% trypan blue solution (Sigma Aldrich, UK) was diluted 1:1 in phosphate buffered saline (PBS), and 100µL gently pipetted up and down to evenly disperse the cell pellet. The cell suspension was incubated for 3 minutes at room temperature to allow the dye to be absorbed into the damaged cells, as a longer incubation time can cause cell death and influence cell viability. 20µL of the cell suspension was added to the haemocytometer, with the unstained (viable) and stained (non-viable) cells counted separately under a light microscope (Strober, 1997). To calculate the total number of cells per ml, the results were multiplied by a factor of 2 to account for the 1:1 dilution factor of trypan blue and the following equation was used to determine cardiomyocyte viability:

$$\text{Cell viability \%} = \frac{\text{total number of viable cells per millilitre of aliquot}}{\text{total number of cells per millilitre of aliquot}} \times 100$$

Each experiment was completed in triplicate, both in plateau and exponential growth phases. Statistics were analysed with SPSS. Paired t-test was used to determine the differences

between doxorubicin doses in comparison to vehicle and control, with statistical significance recognised when $p<0.05$.

2.4 Analysis of cellular growth, behaviour and morphological change by xCELLigence real-time cell analyser (xCELLigence RTCA)

The xCELLigence DP16 RTCA (Agilent technologies., San Diego, CA, USA) is an *in vitro* impedance-based cell analysis system that allows changes in cell survival, morphology and drug response to be evaluated on adherent cell lines in real time using a 16 well electrode plate (E-plate) (Ke et al., 2011, Martinez-Serra et al., 2014). The E-plate incorporates microelectrode sensors, located on the well bases, to record highly sensitive variations in electrical impedance which are converted into cell index values. In wells containing only medium the electric current can flow freely, thus completing the circuit between the electrodes. However, as cells adhere to the well bases, undergoing cell division, growth and distribution, the current flow between the electrodes is impeded and the impedance increases. Conversely, if cells die, reduce in size, or become detached, the impedance will decrease. Through continuous, automatic impedance readings, the xCELLigence monitors these cellular changes, transforming them into graphical form for interpretation and comparison (Ke et al., 2011, Martinez-Serra et al., 2014), allowing cellular perturbations initiated by chemotherapy exposure to be determined.

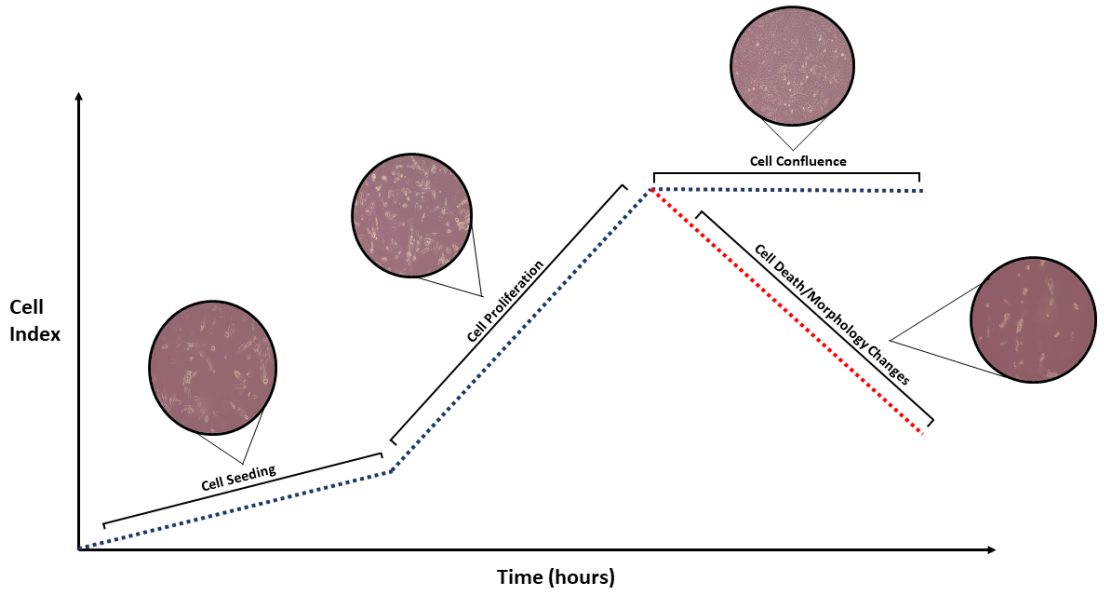


Figure 2.1 Principles of the xCELLigence impedance-based technology. Changes in cellular proliferation, viability and morphology produce impedance measurements, which are represented as cell index over time.

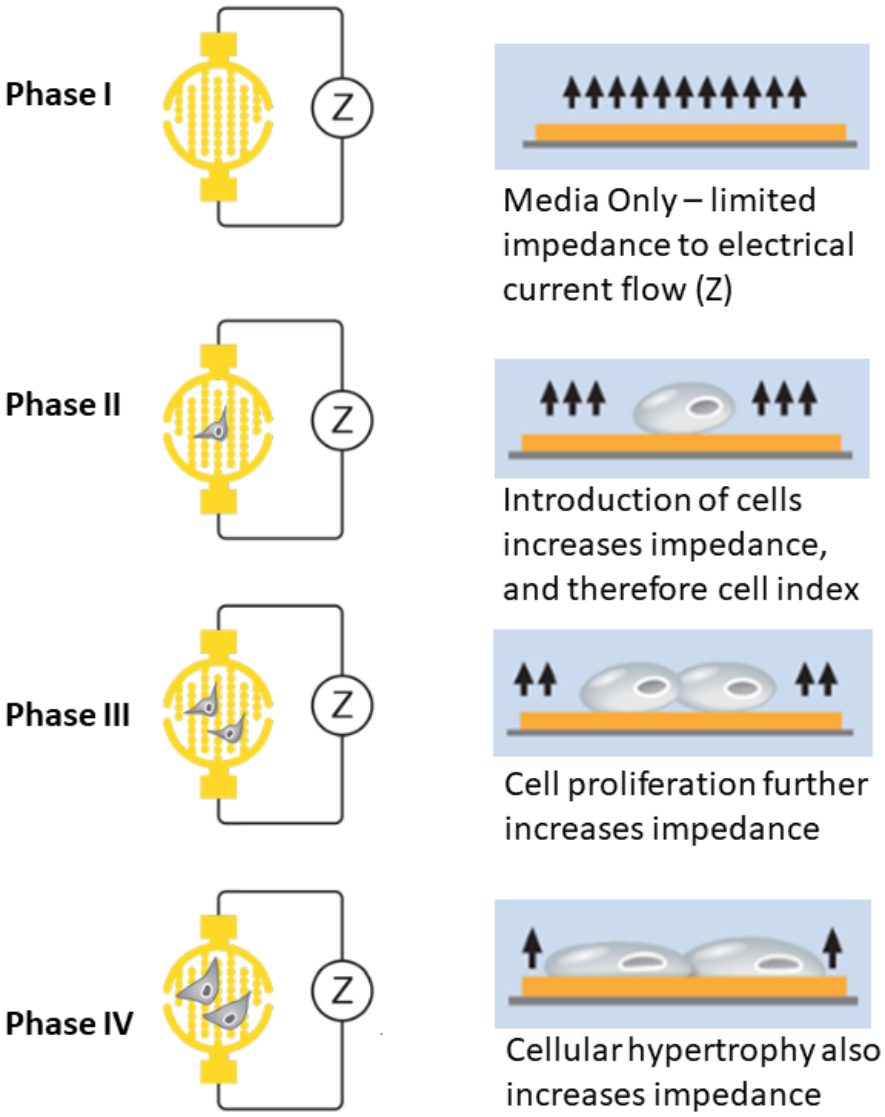


Figure 2.2 **Principles of electrical impedance applied by the xCELLigence technologies to detect changes in cellular adherence, growth and morphology.** Image adapted from ACEA Biosciences Inc., Aglient.

2.5 Evaluation of chemosensitivity of human AC10 cardiomyocyte cells to doxorubicin

The xCELLigence RTCA software and experiments were set-up as per manufacturer's guidance (ACEA Biosciences, San Diego). The plate layout was configured with details including cell type, cell number and compounds to be added to each well. The length of the experiment, combined with the frequency and total number of impedance readings scheduled be taken (defined by sweeps) were defined within the experimental schedule.

For experiments modelling adult cardiomyocyte exposure to doxorubicin, AC10 cells were treated in the plateau phase of growth, with the xCELLigence technology clearly displaying this phase in real-time during the experiments.

Prior to cell seeding, 100µL of DMEM/F12 media (containing 10% FBS, 2 mM glutamine) was added to each of the wells within the 16-well E-plate, and this allowed to equilibrise at room temperature within the laminar flow hood. The E-plate was inserted into the xCELLigence system within the incubator, and a background reading taken. AC10 cells were prepared, suspended in DMEM/F12 media and cell numbers calculated (see section 2.1.1). All experiments were conducted in plateau; with cells seeded at an initial density of $1x10^5$ cells per well in complete DMEM/F12 media to a final volume of 200µL. The E-plates were inserted into the xCELLigence instrument within the incubator, under physiological conditions of 37°C with 5% CO_2. The RTCA system measured impedance readings at 30-minute intervals, with results plotted as cell index values on the xCELLigence software. When the cardiomyocytes reached plateau phase of growth (between 24 and 72 hours), the media was changed, and doxorubicin treatment was added to each well either in duplicate or quadruplicate wells per treatment, depending upon experimental aims and design.

Cell medium was changed daily, either maintaining the same concentration of doxorubicin in the treatment wells, or replicating doxorubicin's half-life by extracting half of the treatment media (100µL) and replacing with 100µL of fresh doxorubicin-free culture media to maintain the 200µL volume. Cells were monitored in real-time throughout the duration of the experiments to observe the time and concentration effects of compound treatment.

Data analysis was completed using the xCELLigence software system, with raw impedance values extracted for analysis. The cell index was initially normalised to the time point prior to drug addition (represented as Δ, i.e. baseline), and then normalised again to the vehicle control (0.1% DMSO) (represented as ΔΔ, i.e. results were double-normalised). The results were then expressed as a percentage change relative to baseline, which was considered 100%.

a) *Normalisation to baseline* (Δ%) = $\frac{Treatment\ Impedance}{Baseline\ Impedance}$ x 100

b) Double normalisation to vehicle control (ΔΔ%) = Δ% of sample – Δ% change of control

Statistical analysis was completed using one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test using GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, California USA). Statistically significant differences were defined as $P<0.05$.

2.6 Primary neonatal cardiomyocyte isolation and preparation

A protocol was developed to extract and isolate primary neonatal rodent ventricular cardiomyocytes from Wistar rats. This protocol detailing the precise methods for sample preparation for the culture and differentiation of the neonatal rodent cardiomyocytes is presented in Appendix A. These cardiomyocytes were isolated, counted and prepared for analysis on the xCELLigence DP16 RTCA. Cardiomyocytes were seeded at a density of 30,000 cells per well, to a total volume of 200µL DMEM. Cardiomyocytes were cultured for 48 to 72 hours to reach their plateau phase of growth before treatment with doxorubicin. Doxorubicin concentrations ranging from 50nM to 500nM were prepared and administered. The time and concentration effects of doxorubicin treatment upon the primary neonatal rodent cardiomyocytes was evaluated using the real-time xCELLigence technology with cell index readings taken at 15-minute intervals and recorded for analysis. The cell index was normalised to time of treatment prior to drug addition (Δ), and then normalised to the vehicle control (0.1% DMSO in DMEM) (ΔΔ%).

2.7 Analysis of gene expression of AT1R following exposure of human AC10 cardiomyocytes to doxorubicin

Reverse-transcriptase polymerase chain reaction (RT-PCR) was used for gene expression analyses to evaluate the presence of specific genes or receptors within samples.

2.7.1 RNA isolation, quantification and analysis of purity prior to gene expression analysis

AC10 cardiomyocytes were seeded in duplicate 6-well plates at a density of 300,000 cells per well and cultured until reaching the plateau phase of growth. Cells were subsequently treated with sub-therapeutic doxorubicin concentrations (50, 100, 250, 500nM) in duplicate wells until the required exposure time, at which point the doxorubicin-containing media was removed to terminate the doxorubicin exposure. Cells were immediately washed with HBSS to remove residual cell debris. Total RNA was extracted from the cells in each well using the protocol RNeasy Mini Kit Qiagen®, according to the manufacturer's instructions (Qiagen, Netherlands). The purified RNA produced was subsequently transferred to RNAse-free PCR microcentrifuge tubes, then immediately frozen at minus 80°C to protect the samples and stored for future experimental use.

The quality and quantity of isolated RNA was evaluated using the NanoDrop® Spectrophotometer ND-1000 (Nanodrop Technologies), with the absorbance ratio 260nm/280nm and 260nm/230nm used to assess the RNA nucleotide purity and protein content, respectively. A 260/280 ratio of >2.0 was considered pure RNA, and 260/230 ratio of 1.8-2.2 being optimal. All samples satisfying these purity criteria were accepted for reverse transcription.

2.7.2 Semi-quantitative reverse transcription for gene expression analysis

RNA samples were reverse transcribed to cDNA using the One Taq® One-step reverse-transcriptase polymerase chain reaction (RT-PCR) kit, according to manufacturer's instructions (New England BioLabs). Each reaction included 1µg RNA in a total 25µL reaction volume, with the reaction conducted using a Techne Prime thermocycler. The conditions for cDNA synthesis and subsequent gene amplification are provided in
Table 2.1, with annealing temperatures optimised for the gene amplified.

Table 2.1 Reverse transcription PCR conditions

Stage	Temperature (°C)	Time (minutes)	Cycles
Reverse Transcriptase	48	15-30	1
Initial Denaturation	94	1	1
Denaturation	94	0.25	40
Annealing	50-65	0.5	
Extension	68	1	
Final Extension	68	5	1
Hold	4	∞	∞

2.7.3 Primer design for RT-PCR

For semi-quantitative PCR, gene expression normalisation was calculated using Glyceraldehyde 3- phosphate dehydrogenase (GAPDH) as the housekeeping gene. Gene-specific primer pairs were designed using Thermo-Fisher Scientific OligoPerfect Primer Designer, with the primer sequences presented below in **Table 2.2**.

Table 2.2 Primer sequences used for RT-PCR

Gene	Primer	Sequence (5'-3')
AT1R	Forward	GAT GAT TGT CCC AAA GCT GG
	Reverse	TAG GTA ATT GCC AAA GGG CC
GAPDH	Forward	GCA CCA CAC CTT CTA CAA TG
	Reverse	TGC TTG CTG ATC CAC ATC TG
HPRT	Forward	TTG CTT TCC TTG GTC AGG CA
	Reverse	ATC CAA CAC TTC GTG GGG TC
RPL13A	Forward	CCT GGA GGA GAA GAG GAA AGA GA
	Reverse	TTG AGG ACC TCT GTG TAT TTG TCA A
TBP	Forward	GCA AGG GTT TCT GGT TTG CC
	Reverse	GGG TCA GTC CAG TGC CAT AA

2.7.4 Agarose gel electrophoresis

DNA amplification products were analysed by separation through a 1% (weight/volume) agarose gel, containing 0.01% ethidium bromide. Samples were combined with 10% loading dye (30% glycerol, 0.25% bromophenol blue) to allow sample visualisation and loaded into the gel alongside a DNA ladder (QuickLoad 100 bp DNA ladder, New England Biolabs). Electrophoresis was performed in Tris-Acetate Ethylenediaminetetraacetic acid (EDTA) buffer (TAE; 40mM Tris, 20mM acetic acid and 1mM EDTA) at 100V for approximately 1 hour. The ethidium bromide-stained gel was examined and photographed under UV light using Biorad® MP ChemiDoc. The bands revealed under UV light correspond to the size of each DNA fragment expressed within the sample and measured against the DNA ladder.

2.7.5 Real-time quantitative RT-PCR (qRT-PCR)

Following RNA quantification using the NanoDrop® Spectrophotometer, reverse transcription to synthesise cDNA was completed by adding 1µg RNA to a sterile Microcentrifuge tube for each cell sample and made up to 12.7µL volume using nuclease-free water. 7.3µL of a prepared 'master mix' solution, consisting of 4µL reverse transcriptase buffer (Promega), 2µL deoxyribonucleotide triphosphate (dNTP) mix (Bioline), 1µL oligo deoxythymine (dT) (Promega) and 0.3µL Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase (Promega), was subsequently added to each sample of RNA to produce a total volume of 20µL.

Each reaction mix was incubated at 65 °C for 5 minutes, then 37 °C for one hour, and finally 100°C for 10 minutes. The total volume for each sample was made up to 100µL by adding 80µL of nuclease-free water.

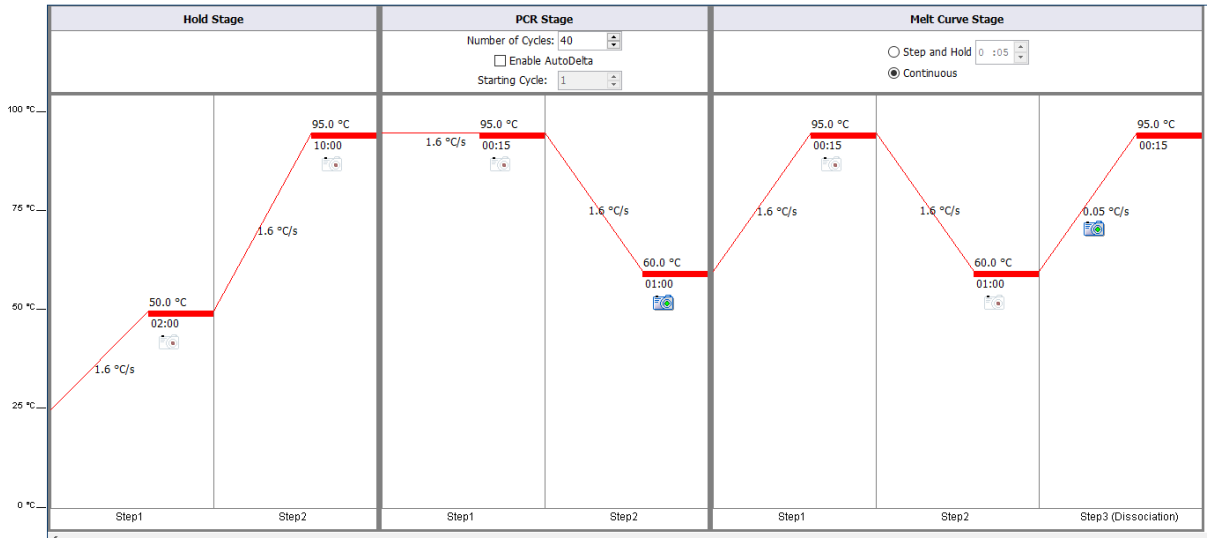
A new 'master mix' for each gene of interest was synthesised by adding 0.8µL of a forward and reverse primer mix (see **Table 2.2** for primer sequences), to 5µL of SYBR green and 2.2µL nuclease-free water. The resulting mix was upscaled to run each RNA sample in triplicate, and included a "no-template control" (wells without cDNA) also completed in triplicate. 8 µL of each gene master mix was loaded onto a 384 well plate, with 2µL of each cDNA sample (synthesised above) added in triplicate to corresponding wells. Reactions were carried out in a total reaction volume of 20 µL. The plate was centrifuged at 1000 xg for 20 seconds before being inserted into the QuantStudio^{TM} 12K Flex Real-time PCR System (Thermo Fisher Scientific). The following qRT-PCR application program was used for all samples (**Figure 2.3**): 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. Fluorescence was acquired at the end of each cycle. After completion of the amplification cycles, a melting curve was determined for each sample by incrementally increasing the temperature from 55-95°C, this confirmed that a single amplicon had been generated for each qRT-PCR reaction. Prior to completing the full gene expression analyses, standard curve controls were produced to check for primer efficiency, stability and exclude technical errors. Five testing concentrations, with a 1:5 serial dilution factor, were initially created for each gene of interest and ran in triplicate. Amplifying different amounts of same cDNA template sample produced a proportional dose-response curve through calculation of the mean Ct values, and concurrently established the detection limit for each gene. The standard curve controls were used to evaluate the reaction efficiency (90-110%), whilst the melt curve peaks observed for impurities and primer-dimers. For full SYBR gene expression quantification analysis, each replicate was completed in triplicate, using separate qRT-PCR runs and RT-cDNA reactions.

Within the literature GAPDH has been identified as a translational suppressor of AT1R expression (Backlund et al., 2009), therefore this study aimed to evaluate this by determination of other 'housekeeping' genes as comparators for calculation of gene expression. The genes GAPDH, 60s ribosomal protein L13A (RPL13A), TATA-binding protein (TBP) and Hypoxanthine guanine phosphoribosyl transferase (HPRT1) were therefore selected as reference genes for qRT-PCR. The reference genes provide a direct control sample for the subsequent qPCR reactions, therefore, identifying a stably expressed reference gene under

the specified reaction conditions, which is essential to accurate gene expression quantification. Amplification of serial cDNA dilutions promoted the assessment of primer efficiencies and established the expression stability for each reference gene. For these analyses, cDNA samples synthesised from AC10 cardiomyocytes pre-treated for 24hrs in culture with varying sub-therapeutic doses of doxorubicin (50nM, 100nM, 250nM and 500nM) were used. 'Control' (media only) and 'vehicle control' (0.1% DMSO dissolved in media) cDNA samples were also produced. The DNA samples were evaluated for purity using Nanodrop with 260/280, with all samples recording a ratio >2 indicating high-quality DNA. These results are presented in Appendix B.

Following completion of the qPCR amplification cycles, the fluorescent properties of SYBR green permits melt curve analysis. The SYBR green fluorophore naturally binds to double-stranded DNA (dsDNA), emitting a fluorescent signal. Incrementally heating the samples from 60 to 95°C causes the dsDNA contained within to denature, producing single-stranded DNA (ssDNA). The dsDNA dissociated from the fluorescent dye, with ssDNA is unable to bind the SYBR green and is therefore represented by a decrease in sample fluorescence. These changes were plotted on a curve of temperature (°C) against change in fluorescence, with the melting temperature (Tm) calculated at the point which 50% of the dsDNA becomes denatured. DNA samples producing a smooth, single peak indicate the synthesis of a single and specific amplicon from qPCR. Whereas curves with multiple peaks suggests either the presences of impurities, the formation of primer dimers or the amplification of non-specific products.

Figure 2.3 Quantitative RT-PCR (qRT-PCR) reaction conditions



Mean threshold cycles (Ct) values and standard deviations for the angiotensin II receptor type-1 (AT1R) gene qRT-PCR analysis were determined by the Quant Studio software. Relative gene expression levels were calculated using the Livak ΔΔCT method which normalises the target gene (AT1R) to the stably expressed reference gene (RPL13A), then calculating the fold change of these normalised coefficient Ct values relative to control (untreated cells). Completing the $2^{\Delta\Delta}$Ct method, these normalised results were expressed in terms of 'fold change', relative to the experimental control which represented untreated (media only) AC10 cardiomyocytes.

2.7.6 Statistical analysis of qRT-PCR results

Prior to statistical analysis, the data expressed as 'fold change' was converted into log2, transforming the data into a normal distribution. To evaluate for significant differences within both the doxorubicin concentration and the exposure time data, mixed ANOVA was completed, complemented by analysis of homogeneity of variance, Brown-Forsythe and Welch's tests to assess the groups for equal variance (i.e. null hypothesis is the groups have approximately equal variance). Post hoc testing with Tukey and Bonferroni was performed in the cases of equal variance, otherwise, in the cases of unequal variance, the Games-Howell test was utilised. P values <0.05 were considered statistically significant, leading to rejection of the null hypothesis, specified as no difference in AT1R expression between groups. Data analysis and graphs were completed in GraphPad Prism version 8.0.

2.8 Protein expression analysis of ATR1 in human AC10 cardiomyocyte cells following exposure to doxorubicin

Western blotting methodology was selected to determine protein expression in cardiomyocytes treated with sequential sub-therapeutic concentrations of Doxorubicin (50, 100, 250, 500nM). AC10 cells were initially seeded in 6-well plates at a density of $3x10^5$ cells per well and incubated in DMEM until the log phase of growth, based upon previous growth curve analysis, was achieved. Samples were then treated with doxorubicin concentrations, with each concentration added to two wells, leaving two untreated wells per plate to act as experimental controls. Duration of cellular exposure to doxorubicin ranged from 4 and 96 hours, to recapitulate clinical pharmacokinetic data.

Following doxorubicin treatment, cells were washed with phosphate-buffered saline (PBS), then lysed using 175µL MRC lysis buffer (including phosphate and protease enzymatic inhibitors) per well to ensure solubilisation of proteins. Lysis buffer-covered cells were scraped

into separate Microcentrifuge tubes for each drug concentration. As the AT1R is located within the cell membrane, cell samples were sonicated for a total of 30 seconds to break open the cellular structure and expose the receptor prior to western blot analysis.

Bradford assay analysis was used for protein quantification of the cell lysates, (Bradford, 1976). Samples were vortexed, with 10µL of solution from each sample added in duplicate to a 96-well plate. Protein standards were also added in duplicate to the 96-well plate for relative protein concentration determination. 200µL of Bradford reagent (B-rad protein and di-ionised water 1:5) was added to each well and left for 5 minutes. Absorbance was read at 595nM using the Multiskan Go spectrophotometer plate reader (Bonjoch and Tamayo, 2001). These absorbance readings were used to calculate the protein concentration of each sample, together with the di-ionised water and loading dye (50% 2x laemelli sample buffer combined with β-mercaptoethanol) for a 30µL sample to be loaded onto a gel. After vortexing, samples were heated at 95°C for five minutes to denature the proteins, then stored on ice prior to western blot analysis.

Tris-glycine Sodium Dodecyl Sulphate (SDS)-Polyacrylamide 10% loading and 5% stacking gels of 1mm thickness, were assembled and placed within an electrophoresis chamber. 4µL of Prestained Protein Ladder (ab116028, Abcam) was added to the first well to determine the sample molecular weights, then each sequential well was loaded with 30µL of total protein lysate per lane. Running buffer was added to the chamber, ensuring sample coverage, and electrophoresis was conducted at an electric potential of 150V for approximately 60 minutes, or until the dye reached the bottom of the gel, to confirm protein separation.

Samples were then transferred from the electrophoresis gel to a methanol-soaked polyvinylidene fluoride (PVDF) blotting membrane, Amersham Hybond (GE Healthcare Life Science). Using a wet transfer method, a transfer sandwich was arranged as: sponge – filter paper – membrane – gel – filter paper – sponge arrangement soaked in transfer buffer containing 20% methanol. This arrangement facilitates the transfer of negatively charged proteins towards the positive electrode, with the blotting membrane binding the travelling proteins before they reach the positive electrode. The electrophoresis transfer tank was with covered ice during transfer to maintain a low temperature (approx. 4°C), with a 300mA current supplied for 60 minutes (Towbin et al., 1979, Mahmood and Yang, 2012)

Following protein transfer to the Hybond membrane, the membrane was briefly washed with Tris-Buffered Saline (TBS) to remove the residual transfer buffer, and then immersed for one

hour with 25mL 5% skimmed milk powder dissolved in TBS and 0.1% Tween®-20 (TBS-T) at room temperature. This process blocked the membrane, thereby preventing non-specific antibody binding.

For evaluation of the AT1R, the primary Anti-Angiotensin II Type 1 Receptor Antibody (Abcam ab124734) concentration was optimised for 1:250 dilution in 1% milk solution, and the membrane was subjected to overnight coating with agitation at 4°C. After three x 15 minutes washes with 25mL TBS-T to remove unbound antibody residues, the western blotting membrane was exposed to the respective species-specific secondary antibody and incubated for one hour. The secondary antibody for ATR1 was horseradish peroxidase-labelled affinity-purified anti-rabbit IgG (H+L) (Vector®), optimised to 1:1500. With the optimised secondary antibody applied to the membrane, the three x 15 minutes 15mL TBS-T washes were repeated to again promote residual antibody removal.

Monoclonal Anti-β-Actin antibody produced in mouse (Sigma-Aldrich®) was selected as loading control to ensure equal loading of the gel and provide an accurate comparator for quantitative protein analysis. The same membrane used for AT1R analysis was first stripped using acid-stripping buffer, then blocked again using 5% milk solution combined with TBS-T and gently shaken for one hour. The process for primary and secondary antibody application was repeated as for the AT1R exposure, with the primary antibody optimised to 1:4000 dilution and the respective secondary anti-mouse antibody concentration to 1:2000.

Following the three TBS-T washes, the membrane was treated with 3mL of enhanced chemiluminescent detection reagent (ECL) mixed just prior to application. The membrane was covered for three minutes to reduced light exposure and degradation prior to detection of proteins. The membrane was then drained of the excess ECL mixture and placed within a plastic film prior to exposure. Detection was performed using either Biorad® MP ChemiDoc or Li-cor® Odyssey® Fc imaging system, with approximate exposure time 180 seconds. The results were digitalised and saved. The protein ladder was used to determine the molecular weight of visible bands for the β-actin and AT1R treated membranes, with predicted molecular weights 42 kDa and 41 kDa, respectively.

The single protein bands revealed on western blotting were subsequently quantified using Fiji ImageJ software (Fiji, Image J 1.52, National Institutes of Health, USA). Analysed within their respective sample lanes, signal intensity was analysed with densitometric quantification. The darker signals produced the highest peaks, whilst the broader signals were represented by

wider peaks. To provide the final signal quantification value for statistical analysis, the background signal emitted from the membrane subtracted from each sample reading by drawing a baseline under each peak.

Statistical analysis was completed by calculating the fold change between the target antibody (AT1R) and the reference antibody (β-actin). The delta control value was then subtracted from the delta treatment value to produce the fold change. All values are reported as mean ± SEM, with statistical analysis completed using Prism 8 (GraphPad, San Diego, CA, USA) and SPSS (Version 25 SPSS, Inc., Chicago, IL, USA). Statistical significance was calculated by multivariate analysis of variance (MANOVA) to determine the interaction between values for full data sets. ANOVA used to determine statistical significance between values within each group. Statistical significance was indicated by $p<0.05$.

2.9 Analysis of cellular expression of ATR1 in human AC10 cardiomyocytes, determined by immunofluorescent staining

Immunofluorescence was performed to evaluate the sub-cellular and translocation of the AT1R in AC10 cardiomyocytes exposed to varying concentrations of doxorubicin. Glass cover slips were initially sterilised in 100% ethanol, before being positioned flat within individual wells of a 24-well plate. The plate was exposed overnight to UV light to complete the sterilisation process. AC10 cardiomyocytes were then seeded at $1x10^4$ cells per well within 500µL of DMEM, then incubated overnight at 37°C to promote adherence to the glass cover slips. The following day, the media was extracted and replaced with DMEM containing sequential sub-therapeutic doses of doxorubicin (i.e. 50, 100, 250, 500nM), except for wells used as experimental control (DMEM only) and vehicle (0.1% DMSO, diluted in DMEM). The AC10 cells were exposed to doxorubicin for 24 hours before commencing the immunofluorescence protocol.

The media was removed, and the cells washed twice with PBS. The cells were then fixed with 300µL ice cold methanol at -20°C for 30 minutes. After removing the methanol, cells were again washed twice with PBS for 5 minutes each, and 0.1% Triton X-100 in PBS was added for 10 minutes to permeabilise the cells. After washing with PBS, 4% bovine serum albumin (BSA) in 0.1% Tween 20/PBS blocking solution was added to each well for 30 minutes at room temperature. The anti-AT1R polyclonal antibody (Abcam 124505) or the recombinant anti-AT1R monoclonal antibody [EPR3873] (ab124734) was diluted in fresh blocking solution at either 1:100 or 1:200 and incubated for one hour at room temperature in a humidity chamber.

The cells were subsequently washed 3 x 5 minutes with PBS to remove excess primary antibody. Goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluro 568 (ThermoFisher, UK) was diluted in fresh blocking solution at 1:400 and applied to the cells for 30 minutes at room temperature. Cells were washed in PBS 3 x 5 minutes to remove excess secondary antibody and mounted in VECTASHIELD containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, San Francisco). The coverslips preserving the adherent cells were carefully removed from the wells and applied to microscope slides using mounting media. Cell staining was evaluated using a HAMAMATSU Digital Camera C11440 ORCA Flash 4.0 microscope. The digital images were taken, optimised, and analysed using Leica Application Suite (LAS X, Wetzlar, Germany).

To best establish the influence of doxorubicin treatment upon AT1R expression, multiple measures of fluorescence intensity units were calculated using an algorithm constructed in CellProfiler (**Table 2.3**).

Table 2.3 Immunofluorescence analysis parameters and measurements constructed within CellProfiler pipeline

Analysis Parameters	Measures
Nucleus cell count	Total number
Area occupied by cells	Area occupied, total area, percentage area covered
Image intensity	Mean, median, minimum, maximum, standard deviation, total area, total intensity
Radial distribution	Mean, standard deviation
Primary objects intensity	Mean, median, standard deviation, upper and lower quartiles

Increases fluorescence intensity represents an increase in AT1R expression, with results expressed relative to the untreated control. Following experiment optimisation, the accuracy of intensity quantification was strengthened by calculating the mean immunofluorescence intensity of the individual cardiomyocytes and by also excluding background fluorescence readings. Analysis of the cardiomyocytes under enhanced magnification improved cell delineation and promoted the cellular localisation of AT1R expression.

2.10 Histopathological analysis of cardiac tissue in paediatric cancer patients with anthracycline-induced cardiotoxicity

Caldicott approval from Newcastle upon Tyne Hospitals NHS Foundation Trust was granted to retrospectively review the medical records, histopathology reports and microscope slides of cardiac tissue from patients who had received cardiac transplantation for anthracycline-induced cardiomyopathy between 1998 and 2020. Sixteen paediatric cancer patients were included within the study, with all patients diagnosed with late-onset anthracycline-induced cardiotoxicity based upon clinical history and cardiac imaging. Of these patients, ten were females and six were males. The patients' ages at the time anthracycline treatment ranged from (mean = 3 years, 10 months; median = 1 year, 10 months). The duration between starting anthracycline chemotherapy and cardiac transplantation of anthracycline-induced cardiomyopathy ranged from 8 months to 21 years (mean = 9 years, 3 months; median = 8 years, 5 months). The cumulative dose for anthracyclines was not available in most cases either due to historical records or absence of documentation.

4 µm thick sections of ventricular myocardium were obtained from the explanted hearts, with histology slides prepared for analysis by staining with haematoxylin and eosin. Slides were analysed using light microscopy and applying a pathological scoring system, with the pathologist blinded to the clinical history and anthracycline dose. The samples were evaluated for necrosis, myocytolysis, interstitial fibrosis and replacement fibrosis, as detailed by Bernaba et al. (2010). Lesions were systematically graded using semi-quantitative localisation scoring: focal lesions scored 1, multi-focal lesions 2, and diffuse lesions scored 3.

Heart weights of the collected samples were compared to historic controls (heart weight normalised for the patient's height) using the Zeek equation (Zeek, 1942). Expected heart weight is calculated as indicated below. A paired Student's *t* test was used to compare actual heart weights to expected heart weights (calculated by Zeek equation).

Male: Heart weight (g) = (Body height (cm) x 1.9) – 2.1

Female: Heart weight (g) = (Body height (cm) x 1.78) – 21.6

2.11 Genotyping of ACE in human clinical samples

2.11.1 Isolation of DNA from human buccal samples for prospective genetic analysis studies

Buccal samples were collected for DNA/RNA extraction using SK-15 swabs (Isohelix, UK). 500µL stabilisation BFX buffer (Isohelix) was immediately added to the tube of each swab upon receipt, according to manufacturer's protocol, with these then stored at 4°C prior to batch DNA isolation.

DNA isolation was completed in accordance with the recommended Isohelix BuccalFix Plus DNA isolation protocol, as follows. 20µL of protein kinase solution was added to each buccal swab tube (containing 500 µL of lysis-stabilisation buffer) and the tubes incubated at 60°C for one hour. The solution was transferred into a 1.5mL DNase/RNAase/DNA-free microcentrifuge tube containing 500µL DNA precipitation buffer. The swab head was also placed into a microcentrifuge tube and centrifuged at 12,000 xg for 5 minutes, with the resultant supernatant added to the extracted solution. The combined solution was centrifuged for 10 minutes at 12,000 xg and the supernatant carefully removed. The resulting DNA pellet was resuspended in 50µL of TE solution, centrifuged for 10 minutes at 12,000 xg, and the final DNA-containing supernatant transferred to a sterile microcentrifuge tube. The newly isolated DNA was analysed for purity and concentration using the Nanodrop ND-1000 Spectrophotometer, with the expected DNA yield between 10 to 100ng/µL. DNA samples were either used immediately for PCR amplification, or stored at -80°C for subsequent studies.

Buccal swab samples were collected from healthy volunteers (n=12), from patients treated with anthracycline chemotherapy for breast cancer and lymphoma recruited to the PROACT clinical trial (n=35) and oncology patients (breast cancer, leukaemia and sarcoma) attending the cardiotoxicity clinic (n=6). Project and ethical approval were granted by Newcastle biobank [REC reference: 17/NE/0361, IRAAS project ID: 233551, Application NB-190], with all samples collected following receipt of written informed consent from study participants (see Appendix C for details).

2.11.2 Retrospective genetic analysis of ACE genotype in breast cancer patients treated with anthracycline chemotherapy

2.11.2.1 Identification of eligible patient samples for retrospective genetic analyses.

Blood plasma and DNA samples for retrospective analysis of the genetic polymorphism of ACE and its association with anthracycline-induced cardiotoxicity were identified from those previously obtained for pharmacogenetic studies at Newcastle University. Patients eligible for inclusion were those diagnosed with early-stage breast cancer and treated with anthracycline chemotherapy between 12th March 2002 and 15th July 2011 (Bray et al., 2010, Jamieson et al., 2011, Jamieson et al., 2014, Jamieson et al., 2017). Ethical approval for the use of these samples for the genetic study was granted from Newcastle and North Tyneside Research Ethics Committee, with permission from Newcastle Biobank retrospectively granted to evaluate samples previously obtained from this long-term patient cohort.

Patient demographics, electronic hospital medical records and clinical echocardiography reports were evaluated to identify cases of cardiotoxicity in the 289-patient study (Bray et al., 2010, Jamieson et al., 2011, Jamieson et al., 2014, Jamieson et al., 2017). Cardiotoxicity was defined as a reduction in left ventricular ejection fraction (LVEF) to below 55% by echocardiography, and a greater than 10% reduction in LVEF from baseline. Cases were specified as 'late-onset' if the reduction in LVEF or onset of heart failure symptoms presented more than one year since starting the chemotherapy treatment. From this cohort of 289 patients, 41 patient plasma and DNA isolates were available for analysis.

Applying the above methodology, 81 patients from the 289-patient pharmacogenetic trial were identified as having received an echocardiogram following chemotherapy, and so their cardiotoxicity phenotype could be determined (29 patients with cardiotoxicity, and 52 patients without cardiotoxicity). Therefore, combining these details of known cardiotoxicity status together with plasma/DNA isolate availability, 17 patients were selected for analysis within the comprehensive angiotensin study.

2.11.2.2DNA extraction from patient blood samples for retrospective analysis of ACE genotype.

Blood samples collected as part of the previous pharmacogenetic studies had been obtained in ethylenediaminetetraacetic acid (EDTA)-containing tubes prior to treatment, with plasma separated following centrifugation at 2000 xg for 10 minutes and stored at -20°C (Jamieson et

al., 2017). DNA was extracted in batches for genotyping analysis using QIAmp Maxi Blood kit (Qiagen®,UK) in accordance with the manufacturer's instructions, as detailed in Jamieson et al. (2014). The quality and quantity of isolated DNA was subsequently evaluated using the NanoDrop® Spectrophotometer ND-1000. DNA samples were subsequently stored at -20°C (Jamieson et al., 2014).

2.11.3 Gene amplification of ACE gene by PCR

Analysis of ACE genotype was performed as previously described (Rigat et al., 1992, Yoshida et al., 1995). PCR reactions were conducted using One Taq® RT-PCR reagents, as outlined in **Table 2.3**, and with the PCR primer sequences identified in **Table 2.4**. Reactions were performed as per manufacturers' instructions (New England Biolabs).

Table 2.3 DNA amplification reagents

Reagent	Stock Solution Concentration	Final concentration	Total volume per PCR reaction (µL)
PCR buffer	5x	1x	4.0
Deoxynucleotide triphosphates	10mM	200µM of each dNTP	1.0
Magnesium chloride	25mM	1.3mM	1.0
ACE primer-sense	20µM	0.8µM	0.5
ACE primer-antisense	20µM	0.8µM	0.5
Taq DNA polymerase	5 units/µL	0.2 units/µL	1.0
Nuclease-free water	-	-	7.0
DNA sample	-	-	5.0
Total Volume			*20µL*

Table 2.4 PCR primer sequences for ACE genotyping

Gene	Primer	Sequence (5'-3')	Annealing Temperature (°C)	PCR Product Size (bp)
ACE	Sense	CTG GAG ACC ACT CCC ATC CTT TCT	58	490/190
	Antisense	GAT GTG GCC ATC ACA TTC GTC AGA T		
GAPDH	Sense	GCA CCA CAC CTT CTA CAA TG	60	600
	Antisense	TGC TTG CTG ATC CAC ATC TG		

PCR amplification was performed using a Techne Prime thermocycler. The PCR reaction was as follows: initial denaturation at 94°C for 10 minutes, then 30 cycles of denaturation at 94 °C for 1 minute, primer annealing at the relevant temperature for 1 minute and DNA extension 72 °C for 2 minutes. A final extension at 72 °C for 2 minutes was added and the samples then kept at 15°C prior to analysis.

Following DNA amplification, 5µL gel loading dye (6x, New England Biolabs) was added to each amplified DNA sample, with 10µL of this mixture separated through a 1.5% agarose gel in TBE buffer (as detailed in section 2.7.4).

2.11.3.1 Confirmation of the presence of the insertion genotype of ACE

As a result of preferential amplification of the D allele, approximately 4-5% of samples classified as the I/D genotype are mistyped as D/D (Shanmugam et al., 1993). Therefore, all D/D genotype samples were reassessed using an additional pair of ACE insertion-specific gene primers (Biller et al., 2006). With this further PCR reaction, the I allele produces a 335-bp amplicon, yielding no product in homozygous D/D samples (Shanmugam et al., 1993, Lindpaintner et al., 1995). The PCR reaction was conducted as previously described in section 2.11.3, using the primers specified in **Table 2.5.**

Table 2.5 PCR primer sequences for confirmatory genotyping

Gene	Primer	Sequence (5'-3')	Annealing Temperature (°C)	PCR Product Size (bp)
ACE Insertion-Specific	Sense	TGG GAC CAC AGC GCC CCG CCA C TAC	62	335
	Antisense	TCG CCA GCC CTC CCA TGC CCA TAA		

2.12 Quantification of systemic Angiotensin II levels in human blood plasma

Levels of angiotensin II were determined from the plasma of patients in the retrospective pharmacokinetic and pharmacogenetic study, matched to the respective DNA sample assessed for ACE genotype (Jamieson et al., 2017). Fresh blood plasma samples were also obtained from a cohort of healthy volunteers, to represent a control population. To maintain angiotensin II stability, all blood samples were collected in EDTA-containing vacutainers, gently inverted to ensure anti-coagulation and enzyme inhibition, and immediately placed at 4°C to

minimise angiotensin II breakdown. Blood plasma was isolated by centrifugation at 4°C, aliquoted into small volumes, and promptly stored at -80°C until assayed. Prior to analysis, sample aliquots were carefully brought to 4°C and centrifuged to isolate impurities.

Quantification of angiotensin II in blood plasma was performed using a commercially available ELISA assay, (ADI-900-204, Enzo Life Sciences, Farmingdale, New York, USA). The competitive inhibition assay procedure was performed in accordance with the manufacturer's protocol, with each sample analysed in duplicate, and absorbance determined at 450nm using the Multiskan Go spectrophotometric plate reader. Plasma angiotensin II concentrations were calculated using GraphPad Prism (version 8.0.0). The standard curve of known angiotensin II peptide was established and four-parametric logistic curve fitting was applied to the results, plotting percentage bound versus angiotensin II concentration. To determine levels of angiotensin II in the blood plasma samples, absorbances readings were interpolation onto the standard curve. Final plasma concentrations were reported in pg/mL.

Statistical analysis were completed using GraphPad Prism and SPSS (Version 25 SPSS, Inc., Chicago, IL, USA). To determine the sensitivity of angiotensin II to freeze/thaw cycles, repeated measures ANOVA was applied with Bonferroni correction and Mauchly's sphericity test. Analysis of patient characteristics was compared using independent t-test for continuous variables and Chi-squared test for categorical variables. The association between ACE I/D polymorphism and prognostic factors was evaluated using either Chi-squared testing or Fischer's exact test, with the Hardy-Weinberg equilibrium calculated to provide predictive values for allele frequencies.

2.13 Analysis of relationship between baseline blood pressure and clinical development of anthracycline-associated cardiotoxic heart failure

To establish the relationship between anthracycline-induced cardiotoxicity and blood pressure, a cohort of 289 patients diagnosed with breast cancer were retrospectively evaluated for changes in blood pressure before and during treatment with anthracycline chemotherapy. Following ethical approval granted from Newcastle University biobank (REC reference 12/NE/0395), patients diagnosed and treated for breast cancer at The Newcastle Upon Tyne Hospitals NHS Foundation Trust between 1998 and 2006 were eligible for inclusion within the study. The diagnosis of anthracycline-induced cardiotoxicity within patients was established based upon either, a reduction in left ventricular ejection fraction (LVEF) to below 55% by echocardiography, and a greater than 10% reduction in LVEF from baseline. To classify

the severity of cardiac impairment, the former British Society of Echocardiography (BSE) criteria was applied, categorising cases as either mild (45-55%), moderate (35-45%), or severe (≤35%) based upon cardiac ejection fraction.

For inclusion within this retrospective cohort study, it was necessary for patients to have received anthracycline chemotherapy, without concomitant trastuzumab or liposomal anthracycline preparations. Patients were also required to have received at least one echocardiogram following chemotherapy to evaluate for cardiotoxicity, whilst documented bloods pressure measurements both before and during chemotherapy were also essential inclusion criteria. Clinical data regarding patient demographics, co-morbidities, concurrent medications, details of anthracycline chemotherapy including number of treatment cycles and cumulative anthracycline dose were collected to support statistical analyses. With these inclusion criteria, thirteen patients were deemed eligible for inclusion within the study. At a median follow-up of 17 years, six patients had developed anthracycline-induced cardiotoxicity; three patients with mild heart failure (LVEF 45-55%), and three with severe heart failure (LVEF ≤35%).

2.14 Analysis of the cardiovascular risk profiles in patients treated for breast, prostate and lung cancer

The cardiovascular risk profiles of three groups of patients with cancer were prospectively evaluated to establish the magnitude of cardiovascular risk factors and cardiovascular disease burden within the cancer population. 208 women with a mean age 71.4 years (s.d. ± 8.8 years) previously enrolled in the pharmacokinetics trials were evaluated for current cardiovascular risk factors and cardiovascular risk scores were calculated as of January 2020. Patients were not eligible of inclusion within the analysis if they had a previous diagnosis of coronary artery disease (i.e. angina, myocardial infarction) or cerebrovascular disease (i.e. transient ischaemic attack, stroke). 20 consecutive patients from each of the prostate and lung cancer clinics were interviewed to establish a baseline cardiovascular history, with screening for previous cardiovascular events, cardiovascular risk factors and treatment performed. These details were used to calculate an estimated 10-year cardiovascular risk score of patients using the QRISK3® algorithm (https://qrisk.org/three). In accordance with the QRISK3® risk assessment tool, patients with a previous diagnosis of cardiovascular disease (e.g. angina, myocardial infarction, transient ischaemia attack or stroke) were excluded from the study, as the risk estimate has not been validated for this population. Primary prevention medications for

cardiovascular disease were also reviewed, with indications for lipid lowering therapy assessed in accordance with National Institute for Health and Clinical Excellence (NICE) clinical guidelines [CG181: September 2016].

2.15 Risk stratification of anthracycline-induced cardiotoxicity in paediatric cancer survivors

Ethical approval from Newcastle biobank [REC reference: 17/NE/0361, IRAAS project ID: 233551, Application NB-190] was granted to retrospectively establish a cohort of long-term paediatric (i.e. less than 18 years of age at the time of diagnosis) cancer survivors for assessment of anthracycline-induced cardiotoxicity. 178 patients attending the long-term paediatric cancer survivors' clinic between August 2019 and August 2020 at The Newcastle upon Tyne Hospitals NHS Foundation Trust were identified for inclusion within the study. Patients were evaluated based upon the presence or absence of the cardiotoxicity phenotype, demographics and establish cardiotoxicity risk factors. The variables considered were patient age at chemotherapy treatment completion, female sex, cumulative anthracycline dose, mediastinal radiotherapy, and whole-body radiotherapy.

Electronic clinical records and echocardiography reports were accessed to identify eligible patients. Cardiotoxicity was defined as a reduction in left ventricular ejection fraction (LVEF) to below 55% by echocardiography, or a greater than 10% reduction in LVEF from baseline. In terms of fractional shortening indicative of cardiotoxicity, this was defined based on the previously accepted criteria of fractional shortening (FS) <28% (Kremer et al., 2002, Hudson et al., 2007, Tissot et al., 2018). Each potential cardiotoxicity case was reviewed by two independent cardiology specialists to establish individual cardiotoxicity statuses.

In agreement with the International Late Effects of Childhood Cancer Guideline Harmonization Group, cardiomyopathy risk stratification and surveillance recommendations for survivors of childhood cancer were based upon anthracycline dose (mg/m^2) and chest radiation (Gy) (Armenian et al., 2015). Patients identified with the cardiotoxicity phenotype were therefore evaluated and grouped according to their risk score (see **Table 2.6**).

Table 2.6 Cardiomyopathy surveillance stratification according to The International Late Effects of Childhood Cancer Guideline Harmonization Group (adapted from Armenian et al., 2015)

Risk	Anthracycline (mg/m2)	Chest Radiotherapy (Gy)	Anthracycline (mg/m2) + chest radiotherapy (Gy)	Surveillance Interval
High	≥ 250	≥ 250	≥ 100 and ≥ 15	5-year, consider more frequently
Moderate	100 to < 250	15 to < 35	-	5-year
Low	> 0 to < 100	> 0 to < 15	-	5-year

2.16 Statistical analysis of clinical studies

Statistical analyses were performed using SPSS software (version 26). Continuous variables were represented by mean ± standard deviation (S.D). Significance was determined, after checking for equality of variance using Levene's test, by analysis of variance (ANOVA) test and with students t-test. Data not normally distributed were transformed to logarithmic form and reassessed for normality. Kruskal-Wallis test was used for variables not normally distributed. Categorical variables with groups were compared using Fischer's exact test or chi-squared (χ^2). Associations between the defined endpoints and risk factors were evaluated using Cox multiple regression analysis. In all cases, a p-value of ≤0.05 was considered statistically significant.

Chapter 3. Characterisation of cardiomyocyte toxicological response to anthracyclines

3.1 The adverse effects of anthracycline chemotherapy upon the cardiovascular system

Since their discovery in the 1950s, anthracycline chemotherapy (*e.g.* epirubicin, doxorubicin and daunorubicin) remains one of the most widely used cancer chemotherapies with proven efficacy across a wide range of solid tumours and haematological malignancies in paediatric and adult patients, notably lymphoma, leukaemia, breast cancer and sarcoma. Despite being highly effective anti-cancer agents, anthracycline chemotherapy is the leading cause of long-term cardiovascular disease in cancer survivors (Mulrooney et al., 2009). Consequently, the successes associated with improvements in overall cancer prognosis and treatment are counterbalanced by detrimental life-threatening effects upon the cardiovascular system, culminating in higher rates of cardiovascular morbidity and mortality.

Most commonly in the clinic, patients present with late-onset cardiotoxicity and/or symptomatic congestive cardiac failure, associated with left ventricular dysfunction, several years post-treatment. The timescale for when a patient will present with detectable cardiac functional impairment is dependent upon several physiological and clinical factors. After initial anthracycline-induced cytotoxicity against the myocardium, compensatory mechanisms stimulate ventricular remodelling, with the remaining functional cardiomyocytes and potentially cardiac fibroblasts undergoing morphological and functional changes, to maintain cardiac mass and cardiac output. The fact that cardiomyocytes have a low capacity for replacement and regeneration, implicates a role for activated cardiac fibroblasts and endothelial cells in cardiac remodelling post-cardiac injury in these processes. The process is further facilitated by inflammatory cell infiltration, initiated by the anthracyclines, and accompanying fibroblast proliferation increasing myocardial collagen deposition, which over time translates into cardiac fibrosis. Progressively this cardiac fibrosis stiffens the ventricles and significantly reduces cardiac power output (Goldspink et al., 2003). Consequently, the gradual losses of cardiomyocytes experienced beyond adolescence places added functional strain upon the remaining myocytes, diminishing the cardiac contractile reserve and the capacity to cope with physiological stressors. In support of this, patients who are treated with additional cycles of anthracycline chemotherapy have been shown to develop cumulative

cardiac injury and replacement fibrosis, thus accelerating cardiac functional decline and the presentation of symptomatic heart failure (Olivetti et al., 1991, De Angelis et al., 2016).

3.2 The cellular and molecular mechanisms responsible for anthracycline-induced cardiotoxicity

The clinical presentation of drug-induced cardiotoxicity upon the cardiac system is associated with identifiable changes in organ function and structure, such as cardiac remodelling, electrophysiological disturbances, and cardiac fibrosis. Historically, drug-induced cardiotoxicity has been classified as either type I (myocardial damage) or type II (myocardial dysfunction) to characterise different cardiomyocyte responses to chemotherapeutic agents (Ewer and Lippman, 2005). Type I cardiotoxicity exhibits a dose-dependent relationship encompassing irreversible cardiac damage; with ultra-structural changes on endomyocardial biopsy revealing vacuoles, myofibrillar loss, mitochondrial swelling and dysfunction, sarcoplasmic reticular dilatation, and loss of overall cardiomyocyte structural integrity and ultimately evidence of left ventricular dysfunction (Mortensen et al., 1986, Rowan et al., 1988, Mackay et al., 1994). By contrast, type II cardiotoxicity does not associate with the same ultra-structural changes and cardiac damage as type I, demonstrating reversibility upon drug cessation and no overt loss in cellular integrity or viability (Ewer and Lippman, 2005). The effects of type II cardiotoxicity being attributed to disturbances in molecular signalling pathways rather than cytotoxic effects per se.

It is without doubt that the toxic effects of drugs, particularly anthracyclines, arise because of drug-induced effects occurring at the cellular and molecular level within the myocardium. There are several mechanistic hypotheses proposed by which anthracyclines induce cardiovascular toxicity, affecting primarily cardiomyocytes but also other cell types within the cardiac myocardium. These effects include inhibition of DNA synthesis related to either DNA intercalation and/or DNA topoisomerase inhibition disturbances within genomic and mitochondrial DNA, dysregulation of cardiomyocyte mitochondrial function and cellular bioenergetics, induction of oxidative stress with free radical formation, and impaired calcium signalling affecting myocardial relaxation (Khiati et al., 2014, Cappetta et al., 2018, Renu et al., 2018). This suggests that the aetiology of cardiotoxicity is multi-factorial at the molecular level (Hutchins et al., 2017, Reichardt et al., 2018). Elucidation of these events will both serve to improve understanding of the molecular basis of this toxicity and identify strategies for prediction and mitigation of this toxicity.

It is accepted that the initial trigger for anthracycline-induced cardiotoxicity is the induction of death in susceptible cells within the cardiac myocardium, supported by the observation of cellular morphological features consistent with apoptosis and necrosis following higher dose anthracyclines (Unverferth et al., 1981, Arola et al., 2000). However, the mechanisms responsible for these effects in a largely quiescent cellular population and the relationship of this acute event to late-stage chronic cardiotoxicity and the response of the 'surviving' cells to this chemical insult and loss of neighbouring cells remains to be determined.

3.3 Preclinical cell models for in vitro *evaluation of anthracycline-induced cardiotoxicity*

To strengthen and progress our understanding of cardiotoxicity, proposed mechanisms within the context of structural toxicity and cardiomyocyte injury need to be characterised. Endomyocardial biopsy studies have demonstrated that cardiomyocyte damage is detectable even before evidence of cardiac functional changes or the presentation of symptomatic heart failure, with a dose-response relationship firmly demonstrated. However, the invasiveness of endomyocardial biopsy renders routine histological sampling of clinical samples impractical for characterising molecular mechanisms of anthracycline-induced cardiotoxicity, especially since serial monitoring is necessary (Lefrak et al., 1973, Bristow et al., 1978b). Therefore, utilising a robust and dynamic *in vitro* model, representative of human cardiomyocytes, is essential to evaluate cardiomyocyte for structural changes in response to anthracycline exposure. In the context of anthracycline-induced cardiotoxicity, the type of cardiotoxicity (structural or functional) and the phase of cardiotoxicity (acute vs chronic) are important criteria for consideration.

Historically, there has been a lack of *in vitro* cardiac models that were both clinically and physiologically relevant i.e. human derived cells that exhibit both a cardiac phenotype and are amenable to molecular studies. Such models are essential for detection of drug effects upon cardiac cells, including both structural and functional changes.

3.3.1 Primary rodent cardiomyocyte cell models for evaluating cardiotoxicity in vitro

Several studies have addressed the use of primary human cardiomyocytes in preclinical studies. However, barriers to supply, dedifferentiation in culture, and the existence of a narrow window of cellular viability by which to monitor drug effects, limits their applicability for routine screening (Li et al., 1996, Bénardeau et al., 1997, Bistola et al., 2008, Burridge et al.,

2016, Nguyen et al., 2017). One approach to address this was to utilise rodent cardiomyocytes, which can theoretically be cultured for longer periods *in vitro* and are more freely available.

Accurate prediction of drug-induced cardiovascular effects is essential to establishing cardiac safety and enhancing treatment strategies. A strength of rodent cardiomyocyte models is that they have been well-characterised, providing valuable structural and functional insights into the pathophysiology of anthracycline-induced cardiotoxicity (Podyacheva et al., 2021). During primary cardiomyocyte isolation, additional cell transfer steps and short incubations can be introduced to provide a more cardiomyocyte-specific model and limit confounding factors such as fibrosis or scar, with removal of additional non-cardiomyocyte cells such as fibroblasts (Louch et al., 2011). Beyond the cardio-histological changes reportedly induced by doxorubicin, similar cardiac structural hypertrophic responses have been readily detected within adult rodent cardiomyocytes, which conveys a significant advantage for the investigation of hypotheses (Zhu et al., 1997, Xu et al., 2010, Menaouar et al., 2014, Du et al., 2017). Although the total duration of contraction of newly isolated adult rodent cardiomyocytes may be limited and therefore restrict the functional data available. Nevertheless, important structural manifestations of cardiotoxicity can be identified within primary culture (Mettler et al., 1977, Ellingsen et al., 1993). Finally, rodent cardiomyocyte cell models may be susceptible to tissue culture infections deriving from the early stages of tissue isolation, therefore, steps to prevent infections must be conveyed combined with regular culture monitoring (Louch et al., 2011).

3.3.2 Primary neonatal rodent cardiomyocytes for evaluating cardiotoxicity in vitro

Neonatal rodent ventricular cardiomyocytes (NRVCs) represent an appropriate model for preclinical cardiotoxicity studies. The key features of NRVCs are that in addition to being well characterised, they are readily available, providing high yields of myocardial tissue, and possessing the ability to retain their contractile phenotype in culture for longer durations after initial isolation. An advantage to using this preclinical model is that the early cardiac histopathology findings described in adult humans post-anthracycline chemotherapy exposure, such as myocyte degeneration and loss, cytoplasmic vacuolisation and interstitial oedema, have also been demonstrated in NRVCs (Mettler et al., 1977, Ivanová et al., 2012). However, a practical limitation of NRVCs is linked to their stage of cardiomyocyte development, with cardiomyocytes retaining the ability to proliferate in culture, particularly in response to hormonal stimuli and cellular injury (Naqvi et al., 2014). Modifiable experimental factors such

as the number of days from post-partum to isolation, the cardiomyocyte isolation techniques and culture media components, can also create morphological and ultrastructural differences, especially when compared with adult cardiomyocytes (Simpson et al., 1982, Simpson and Savion, 1982). Furthermore, difficulties with visualising a definite atrioventricular border may mean that isolating ventricular cardiomyocytes from atrial tissue *in vitro* is incomplete. Together, these variables could lead to alterations in the electrophysiological and ultrastructural properties between the cardiomyocyte isolates, and consequently, the NRVC may not fully reflect cardiotoxicity profiling within adult cardiomyocyte populations, especially within functional studies (Bénardeau et al., 1997, Mitcheson et al., 1998, Brette and Orchard, 2003).

3.3.3 Human AC10 ventricular cardiomyocyte cell line for evaluating cardiotoxicity in vitro

AC10 cardiomyocytes (AC10-CMs) are an immortalised cell line derived by fusing human ventricular cardiomyocytes with fibroblasts transformed with SV40 virus and devoid of mitochondrial DNA (Davidson et al., 2005). Although other cell lines with capability for long-term *in vitro* culture exist, such as the HL-1 mouse atrial cardiomyocyte cell line, and the Hc92 rat cardiomyoblast cell line, these cells being non-human have limited use in examining effects of ventricular cardiomyopathies in humans. Conversely, the AC10-CM cell line, despite being in a pre-contractile state, expresses many cardiac specific transcription factors, contractile proteins and possess functional gap junctions (Davidson et al., 2005, Rockley, 2018). As an immortalised cell line, AC10-CMs (and AC16-CMs) are applicable for use in high-throughput screening assays for detection of structural toxicity, however, current data remains limited. Their strength as a preclinical model for evaluating structural cardiotoxicity has been emphasised across a range of *in vitro* studies, with their cellular growth patterns, mitochondrial function, and expression of cardiac markers, utilised to detect cardiomyocyte inflammation and myocardial injury (Rockley and Gill, 2017, Ouyang et al., 2020, Wen et al., 2020). Furthermore, these cell lines have been applied to closely analyse changes in cell viability and cell apoptosis in response to both serial and cumulative drug concentrations, with the subsequent evaluation of cardiac hypertrophy and cardioprotective effects both highly relevant to the study of anthracycline-induced structural cardiotoxicity (Rockley, 2018, Yoon et al., 2018, Chen et al., 2019).

3.3.4 Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CM) for evaluating cardiotoxicity in vitro

The hiPSC-CM model represents a physiologically relevant source of human adult cardiomyocytes, which has the potential to be cultured *in vitro* for extended periods of time, therefore favouring their application for disease modelling and identifying the mechanisms of anthracycline-induced cardiotoxicity (Asnani et al., 2021). Furthermore, 'patient-specific' hiPSC-CMs have been engineered to enhance structural and drug-induced cardiotoxicity analyses, demonstrating a significant advantage over other human derived *in vitro* models (Burridge et al., 2016, Reinal et al., 2023). Previous concerns regarding the hiPSC-CMs displaying an immature phenotype, specifically relating to cardiomyocyte structure, electrophysiology, and metabolism, have been addressed through the use of cell cycle regulators and by modifying the signalling of known cardiomyocyte proliferation pathways (Robertson et al., 2013, Yang et al., 2023). Similarly, protocols to maintain hiPSC-CMs in culture for even longer durations, including co-culture with physiologically relevant non-cardiomyocytes (i.e. fibroblasts, endothelial cells, smooth muscle cells) and 3-dimensional culture techniques, have been established to promote cardiomyocyte maturation and overcome this recognised limitation (Yang et al., 2023).

Whilst these strengths support the use of hiPSC-CMs for *in vitro* cardiotoxicity studies, their higher purchasing/maintenance costs, and reduced overall capacity for high-throughput screening, when compared with other in vitro cardiac cell models such as rodent primary cells and cardiac cell lines, restricts the availability of hiPSC-CMs (Paik et al., 2020). In addition, hiPSC-CMs have demonstrated high cell-to-cell variability and lower experimental reproducibility relative to other cardiomyocyte models, especially as the cells are required to maintain their proliferative capacity in order to reach required phenotypic maturity (Magdy et al., 2018, Ito et al., 2020). Furthermore, hiPSC-CM differentiation protocols can generate variable ratios of atrial and ventricular cardiomyocytes, therefore structural and functional *in vitro* studies intending to recapitulate cardiotoxicity within adult ventricular cardiomyocytes may be restricted by the presence of a strong atrial component. As drug sensitivities and electrophysiological properties differ between the two cell populations, this challenge is important to address, as doing so will enhance the accuracy of cardiotoxicity analyses and improve translation into patient care (Foglia and Poss, 2016, Magdy et al., 2018).

3.4 Kinetics of cellular uptake of doxorubicin in cardiomyocytes and reflection of the clinical situation **in vitro.**

An important criterion underpinning the study of anthracyclines against cardiac cells is the pharmacological handling of the drug, to best recapitulate the clinical exposure scenario. Clinically, after intravenous administration to patients, doxorubicin displays an initial fast distributive half-life of 3-5 minutes, suggesting rapid tissue uptake, followed by a slow elimination phase, reflected by a terminal pharmacokinetic half-life of between 20-30 hours (Eksborg, 1990, Barpe et al., 2010). This in combination with a steady-state distribution volume of 810-1200 L/m^2 is indicative of extensive drug uptake into tissues, including cardiac tissue, giving rise to increased potential for inadvertent toxicities.

The standard treatment regimens for breast cancer prescribes between 4 to 6 cycles of anthracycline chemotherapy, with each dose administrated as a bolus infusion every 21 days. Anthracyclines typically demonstrate linear dose-related pharmacokinetics. Systemically, following extensive distribution into the human plasma and tissues, doxorubicin is metabolised within the liver and reduced to form doxorubicinol. Re-examining the linear pharmacokinetics of doxorubicin, following intravenous administration the drug usually remains for only 7 days, with more than 50% of doxorubicin excreted via the biliary system. Of the remaining doxorubicin, approximately 23% is excreted as doxorubicinol and the remaining metabolites, with renal elimination completing doxorubicin removal from the body (Danesi et al., 2002). It is also postulated that these secondary metabolites may themselves contribute to cardiotoxicity with elevations in doxorubicinol concentrations associated with *in vivo* cardiomyopathy and evidenced by the retention of these metabolites in post-mortem tissue specimens (Stewart et al., 1993, Menna et al., 2007).

In terms of cardiotoxicity, the major risk factor for development of doxorubicin-induced cardiac dysfunction is cumulative drug exposure, with the incidence of clinical heart failure reported to rise exponentially from 5% with a cumulative dose of 400 mg/m^2 to 48% with 700 mg/m^2 (Henriksen, 2018). Further to this, evaluations of continuous versus bolus infusions have revealed peak dose concentration (Cmax), rather than total drug exposure (area under the curve (AUC), may be the stronger influence of anthracycline-induced cardiotoxicity (Legha et al., 1982, Danesi et al., 2002, Ishisaka et al., 2006). Experimental studies have demonstrated that acute cardiomyocyte exposure to concentrations representative of elevated Cmax levels

causes increased cellular apoptosis and myocardial response mechanisms (Arola et al., 2000). Similarly, although the cumulative dose-dependent cardiotoxic effect of anthracyclines is well recognised, the impact of sequential doses of anthracyclines upon the cardiac system is less well known. It remains unclear whether multiple doses generate repeated cellular stresses in cardiac tissue, or whether the mechanistic basis of cardiotoxicity forms a continuum (Arola et al., 2000, Lipshultz et al., 2006). Indeed, there may be evidence of cellular recovery between treatment cycles, with repeated activation and re-activation of these cardiomyocyte injury mechanisms during recurrent dosing, therefore promoting further cellular losses. In addition, cardiac accumulation of anthracyclines could also theoretically perpetuate intracellular cardiotoxicity mechanisms weeks or even months after chemotherapy completion (von Hoff et al., 1979, Stewart et al., 1993). Following doxorubicin administration, the drug concentration rapidly decreases due to its metabolism and elimination, meaning that cardiomyocytes are then exposed to sub-therapeutic concentrations of doxorubicin over a prolonged time.

Another parameter which, in addition to overall drug concentrations and exposures, has an impact upon anthracycline-induced cardiotoxicity is the mechanism by which cells uptake and handle the drug. In tumour cells anthracyclines are believed to enter the cell by a concentration-dependent passive process, thereafter they enter the nucleus to bind DNA, inhibit topoisomerase activity and induce their cytotoxic effects. This mechanism agrees with studies of transmembrane transport of anthracyclines in other cell types, in which accumulation of anthracyclines is also predominantly through passive diffusion of the unionised drug (Bachur et al., 1976, Dalmark and Storm, 1981). Although passive diffusion may play a role in doxorubicin uptake into cardiac cells, various energy-dependent membrane transporters and enzymes have now been suggested to have a role in doxorubicin-induced cardiotoxicity, mechanisms which are also impacted by pharmacogenetic variations (Lal et al., 2010). One such transporter which has recently gained momentum in the context of doxorubicin transport in human cardiomyocytes is organic cation transporter 3 (OCT3, *SLC22A3*), with blockage of its activity shown to slow uptake of doxorubicin into human induced pluripotent stem-cell derived cardiomyocytes (hiPSC-CMs) (Huang et al., 2021). The significance of this in terms of cardiotoxicity and the clinical manifestation of cardiotoxicity remain to be confirmed.

Taken together, this suggests that to recapitulate clinical patient anthracycline exposure *in vitro* and improve our knowledge of anthracycline-induced cardiotoxicity, accurate cardiotoxicity modelling is essential. Presently, there are a lack of such cardiotoxicity studies modelling acute versus chronic cardiotoxicity, especially studies exploring multiple anthracycline dose exposures, serial half-lives, and effects of sub-therapeutic anthracycline concentrations.

3.5 Clinical pathology of late-onset anthracycline-induced cardiotoxicity

An understanding of the cardiomyocyte responses to anthracycline chemotherapy, and the clinical progression towards congestive cardiac failure, has supported the search for measures with even greater sensitivity and reliability to assess sub-clinical toxicity (McGowan and Cleland, 2003). Analysis of endomyocardial biopsy samples from cancer patients treated with anthracycline chemotherapy showed that within four weeks of receiving anthracyclines, cardiomyocytes were replaced by fibrotic change, which increased ventricular stiffness and impaired myocardial contraction, ultimately leading to cardiac ventricular failure (Bristow et al., 1981). The extent of subsequent myocyte damage and loss was found to be proportional to the cumulative dose of anthracycline received, with the highest doses inducing the greatest degree of myocardial damage (Bristow et al., 1978a, Bristow et al., 1978b). However, these studies were conducted in a small cohort of older patients, addressing acute rather than longer-term changes in cardiac tissue. Late-onset anthracycline-induced cardiotoxicity can develop many years after patients have received chemotherapy, with the progressive histological changes throughout this process incompletely defined beyond this initial cardiomyocyte loss and fibrosis. In the study by Bernaba et al. (Bernaba et al., 2010), which evaluated pathological and histological changes in cardiac tissue, obtained following cardiac transplant or autopsy, from a small cohort of adult patients identified with late-onset anthracycline-induced cardiotoxicity, there was no evidence of significant necrosis or mycocytolysis. Instead, interstitial fibrosis was identified in cardiac tissue from all ten patients, and replacement fibrosis in tissue from six patients, which is notably different from other forms of dilated cardiomyopathy (Bernaba et al., 2010). In contrast to other forms of dilated cardiomyopathy, in which hypertrophy is normally present alongside myocardial fibrosis, in these anthracycline-induced cardiotoxicity specimens, no significant cardiac hypertrophy was observed, despite the presence of fibrosis (Bernaba et al., 2010).

As a diagnostic technique, endomyocardial biopsy exhibits high diagnostic sensitivity, but this invasive procedure does convey a high risk of medical complications, therefore is reserved for cases of diagnostic uncertainty rather than serial monitoring (Bristow et al., 1981). Furthermore, the fact that late-onset anthracycline-induced cardiotoxicity can develop many years after patients have received chemotherapy raises issues regards appropriate timings of these biopsies, with the progressive histological changes throughout this process incompletely defined beyond cardiomyocyte loss and fibrosis (Cascales et al., 2013). Through advanced cardiac imaging techniques such as echocardiography and cardiac MRI, understanding of anthracycline-induced cardiotoxicity has advanced at the organ imaging level, with early structural changes within the myocardium identified and related to prediction of subsequent left ventricular ejection fraction (LVEF) decline or progression to congestive cardiac failure. However, the timing of these changes and relationships to the progressive nature of anthracycline-induced cardiotoxicity and histological changes are as yet largely undefined. Furthermore, there are very few studies actually comparing paediatric and adult responses to anthracycline exposure from a pathological and histological perspective, especially in relation to late-onset cardiotoxicity. These are important criteria to consider when evaluating the clinical impact, identification and application of knowledge regarding prediction and management of subsequent cardiac failure. Therefore, greater analysis of histological and pathological changes within cardiac tissue, especially with regards late-onset anthracycline-induced cardiotoxicity, is ultimately required to assist diagnosis, prognosis and management of this cardiac issue.

3.6 Chapter aims and objectives

This chapter aims to analyse the molecular relationship between angiotensin II signalling and anthracycline-induced cardiotoxicity. Using several qualified *in vitro* cardiomyocyte models, the objectives are to determine the underlying mechanisms and structural manifestations of anthracycline-induced cardiotoxicity, evaluating the relationship between the angiotensin signalling pathway and the mechanism of this toxicity. Specifically:

1. Characterisation and qualification of the AC10 human ventricular cardiomyocyte cell line for use as an *in vitro* model for the study of anthracycline-induced cardiotoxicity.

2. Determine the time- and concentration-dependent cytotoxicity of doxorubicin against the AC10 human cardiomyocyte cell line *in vitro*, particularly in a low proliferative or quiescent growth state recapitulating adult cardiomyocytes in the heart

3. Evaluate cellular responses of human AC10 cardiomyocyte cell line and primary neonatal rodent cardiomyocytes to clinically relevant cardiac exposures of doxorubicin, in real-time using the impedance-based xCELLigence cell analyser system

4. Determine the pathological changes of late-onset anthracycline-induced cardiotoxicity in clinical specimens of cardiac tissue from oncology patients.

3.7 Results

3.7.1 Analysis and qualification of AC10 cardiomyocyte growth kinetics in vitro, using MTT assay

The purposes of these initial experiments were to identify the *in vitro* characteristics of the human AC10 cardiomyocyte cell line (AC10-CM), establish their growth kinetics, and optimise the cardiomyocytes to best replicate human cardiomyocyte physiology for future cardiotoxicity studies.

The MTT assay as a methodology (section 2.3) for quantifying cell number was validated by investigating the relationship between cell number, determined manually, and MTT conversion via quantification of absorbance at 550nM. **Figure 3.1** shows an initial linear relationship between cell number of AC10-CMs and absorbance (R^2 value of 0.9658) when cardiomyocytes are seeding at densities between 1000 to 10,000 cells per well. These results demonstrated that by increasing the total number of viable cells, the collective cellular metabolic activity increased, supporting MTT as a viable methodology for determining cell numbers for AC10-CMs grown *in vitro*.

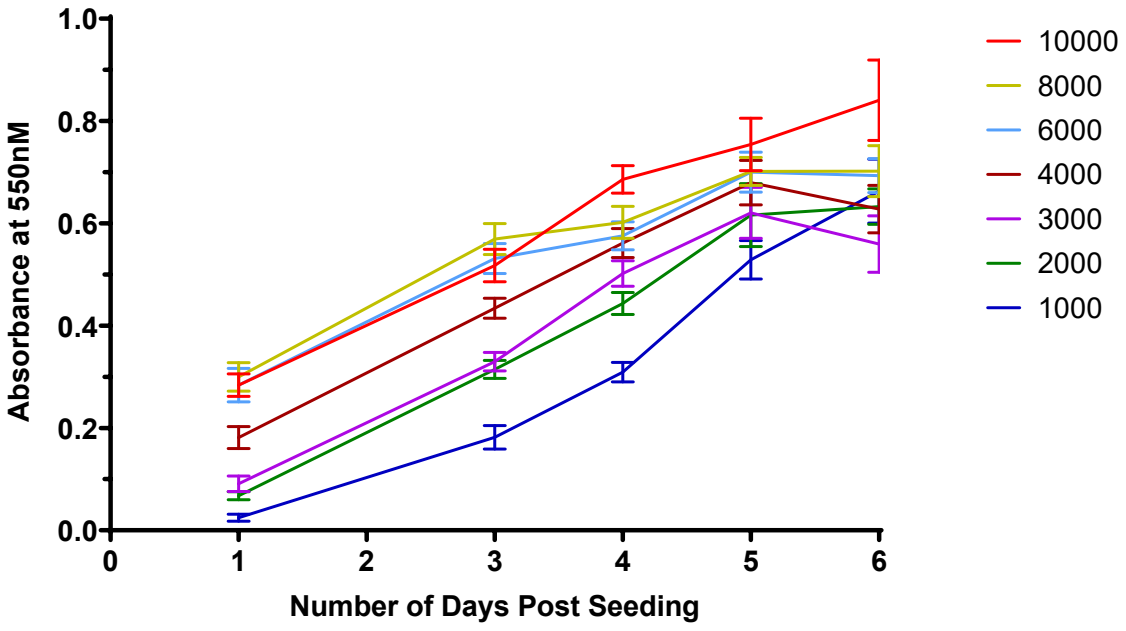


Figure 3.1 **Validation of MTT assay in AC10 cardiomyocytes to assess growth over time using different initial seeding densities.** Data points show mean values ± s.d. of at least three independent experiments.

To assess the suitability of the MTT assay to indicate cellular proliferation of the AC10-CM cell line over a time course, cells were seeded at various densities (1000, 2000, 3000, 4000, 6000, 8000, and 10,000 cells/well) and the absorbance quantified for the MTT assay on six consecutive days (**Figure 3.1**). Cells seeded at 1,000 cells/well shows slow proliferation over the time course and no plateau phase of growth was reached. In contrast, seeding densities from 2000 to 10,000 cells/well showed immediate exponential growth and attaining confluence between 3-5 days post-seeding. At all densities measured, the absorbance measurements were crucially within the limits of assay detection. Subsequently, a seeding density of 2000 cells/well was chosen for MTT studies of cells in the exponential growth phase. Analysis of AC10-CMs whilst in proliferative growth resembles the situation in paediatric cardiac tissue wherein cardiomyocytes do retain a level of proliferative capacity.

To recapitulate the fact that in the adult heart myocyte numbers are known to be relatively stable, due to largely being quiescent and post-mitotic, cell seeding densities and culture durations were assessed to identify those most appropriate for attainment of the plateau phase of *in vitro* cell growth. In this phase of growth, cell death and proliferation rates remain in equilibrium, thereby closely resembling the very low proliferative stability of adult human cardiomyocytes. Analysis of absorbance using MTT assay revealed the absorbance to be relatively stable, indicative of a plateau phase from 5-6 days dependent upon the initial seeding density (**Figure 3.1**). An initial seeding density of 2000 cells and a culture duration of 5 days was chosen for MTT studies in the plateau growth phase.

3.7.2 *Measurement of growth kinetics and cellular response of AC10 cardiomyocytes in realtime, using xCELLigence real time cell analyser*

The xCELLigence RTCA was used to assess the proliferation of AC10-CMs in real-time (section 2.5). The growth of AC10-CMs cells plated at various densities has been previously reported (Rockley, 2018). Accordingly, AC10-CMs were seeded on the cardio E-plate at the higher density of 30,000 cells per well, and the response monitored over a 72-hour period. Cells reached the plateau phase of growth, indicative of the quiescent nature of cardiomyocytes in the adult heart, between 48 and 72 hours (**Figure 3.2**). Therefore the 72-hour time point was established as the optimal time for drug addition when aiming to model adult cardiac drug exposures. To confirm a true plateau phase had been achieved, the media within the wells was changed and the cells were monitored for at least a further 12 hours. This ensured the plateau phase was created due to the formation of a consistent two-dimensional

cardiomyocyte monolayer, rather than growth limited by nutrient consumption within the media.

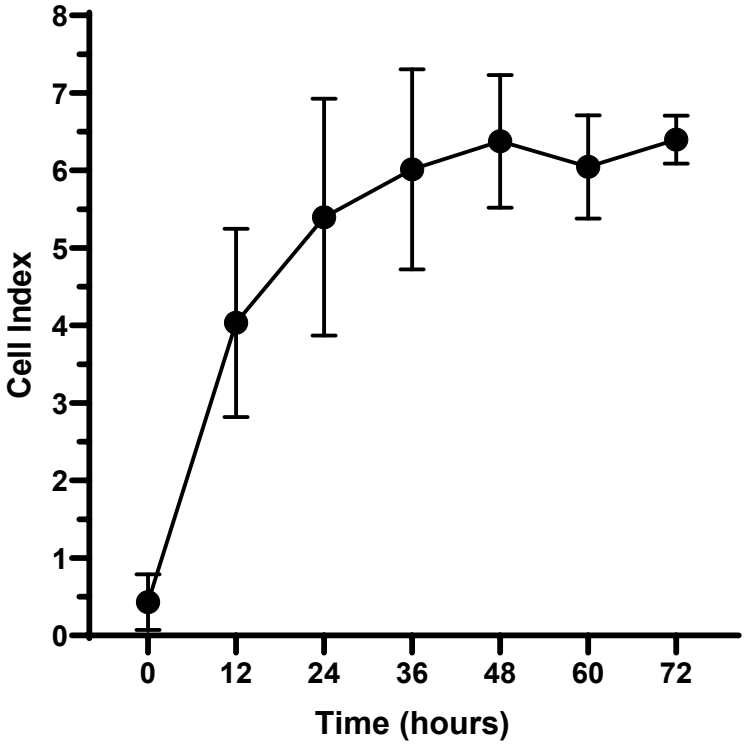


Figure 3.2 Growth kinetics of AC10 cardiomyocytes by xCELLigence real-time cell analysis. xCELLigence traces showing time (hours) vs cell index for AC10-CMs plated at 30,000 cells/well). Data is representative of at least three experimental repeats, data points show average values ± SD.

3.7.3 Measurement of growth kinetics and cellular response of primary neonatal rat cardiomyocytes in realtime, using xCELLigence real time cell analyser

Primary neonatal rodent cardiomyocytes extracted from Wistar rats (section 2.6) were isolated and prepared for analysis on the xCELLigence RTCA (section 2.5). Neonatal cardiomyocytes were seeded at a density of 30,000 cells per well, similar to that used for AC10-CMs, and the response monitored over a continuous 96-hour period. Cells attached to the plate and cell numbers increased consistently over the analysis period **Figure 3.3**. This confirmed the potential for use of the xCELLigence RTCA system for analysis of drug-induced responses in neonatal rat cardiomyocytes, with a time point of 48 hours identified as most appropriate for this purpose.

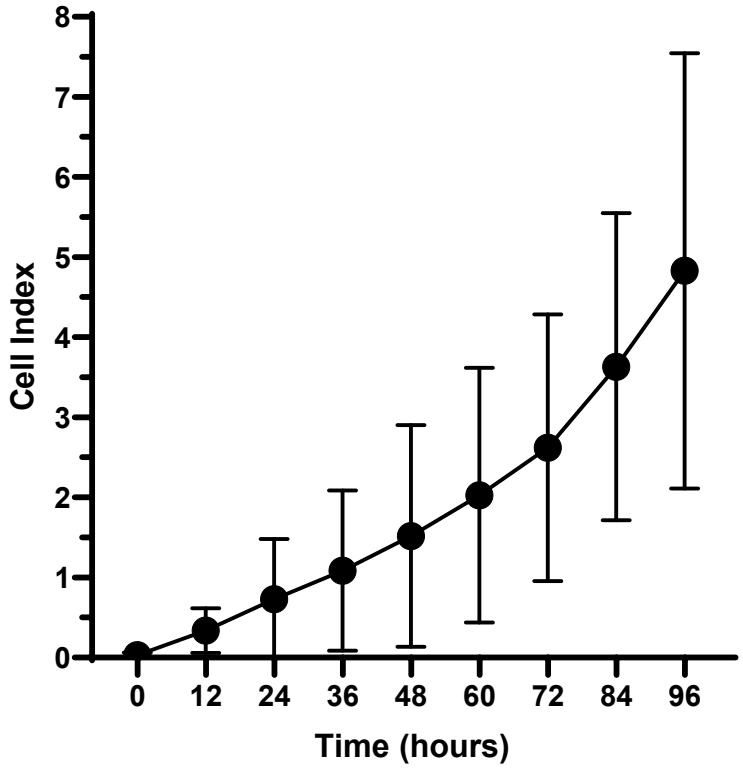


Figure 3.3 **Growth kinetics of primary rodent cardiomyocytes on the xCELLigence RTCA system**. xCELLigence traces showing time (hours) vs cell index for rat neonatal cardiomyocytes plated at 30,000 cells/well). Data is representative of the mean ± S.D. of at least three experimental repeats.

3.7.4 Effects of doxorubicin upon viability of proliferative and quiescent cardiomyocytes, determined by MTT assay

To evaluate the concentration and time-dependent response of AC10 cells to doxorubicin, cellular viability assays were performed. To establish an understanding of the paediatric and adult cardiac response to doxorubicin, the data and optimal experimental conditions formulated from the characterisation of AC10 cardiomyocyte growth were applied. AC10 cells were seeded in 96-well plates at 2000 cells per well and cultivated for either three days until in the exponential phase of growth (paediatric model), or for five days until in the plateau phase of growth (adult model). Cells were exposed to a broad range of serially diluted concentrations of doxorubicin from 0.04 to 10µM and cellular viability determined by MTT assay after 24 hours of drug exposure (section 2.3). The consequent doxorubicin IC_{50} values (representative of decrease in cellular viability by 50%) for AC10-CMs in proliferative and plateau growth phases were and 1.55µM (confidence interval (C.I.) 1.2 to 2.1 µM) and 182.5nM (C.I. 101 to 301nM), respectively (**Figure 3.4**).

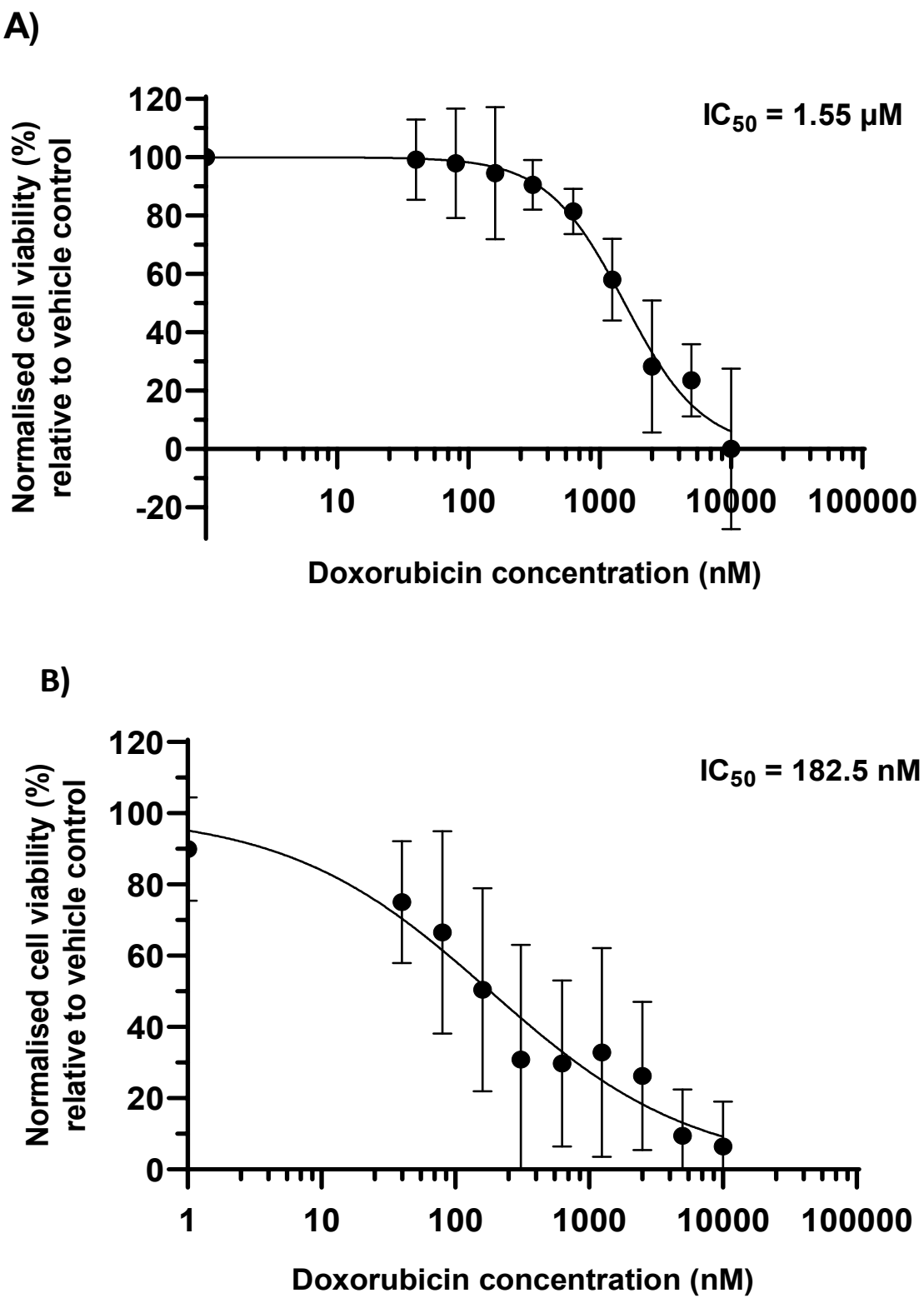


Figure 3.4 **Effect of doxorubicin on viability of AC10 cardiomyocytes in the exponential phase (A) and the plateau phase (B) of growth as determined by MTT assay.** Dose-response curve demonstrating the effect of 24 hours of doxorubicin exposure upon cardiomyocyte viability. Data representative of n=3 ± SEM.

3.7.5 Effects of doxorubicin upon viability of proliferative and quiescent cardiomyocytes, determined by trypan blue exclusion assay

To further characterise the effect of doxorubicin upon viability of AC10-CMs in proliferative and plateau growth and to confirm a lack of interference of the drug upon the mitochondrial mechanism of the MTT assay for this purpose, cellular viability was determined in parallel by the trypan exclusion assay (section 2.3.3). Cellular viability of doxorubicin concentrations ranging from 0.04µM to 10µM were evaluated, with AC10 cardiomyocytes exposed to doxorubicin for 24 hours before analysis. The cellular viability following exposure to doxorubicin measured by trypan blue exclusion was comparable to that determined by MTT assay, with IC_{50} values of 1.1µM (C.I. 0.5 to 2.3µM) and 486nM (C.I 317 to 748nM) calculated for AC10-CMs in exponential and plateau growth phases, respectively (**Figure 3.5**).

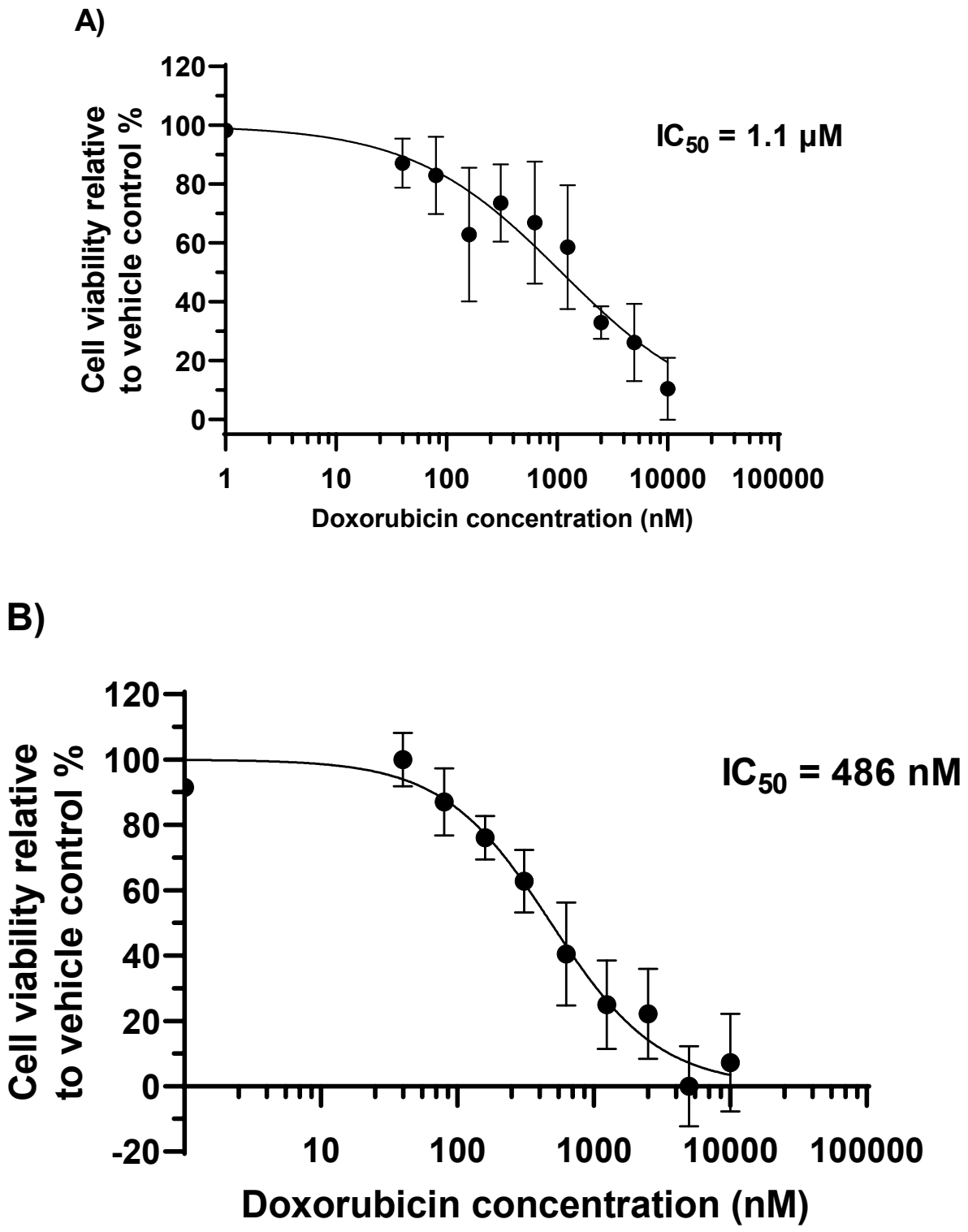


Figure 3.5 **Effect of doxorubicin on viability of AC10 cardiomyocytes in the exponential phase (A) and the plateau phase (B) of growth using trypan blue analysis**. Dose-response curve demonstrating the effect of 24 hours of doxorubicin exposure upon cardiomyocyte viability. Data representative of n=3 ± SEM.

3.7.6 Cellular response of AC10 cardiomyocytes in the presence of decreasing exposures to doxorubicin to reflect pharmacokinetic profile

To evaluate the effects of exposure of doxorubicin upon proliferation, morphology and cellular survival of AC10 cardiomyocytes in real-time, cells were monitored by xCELLigence RTCA over 72 hours. For clinical relevance, doxorubicin doses were based upon the clinical Cmax (1.1µM) and lower pharmacokinetically applicable concentrations (500-50nM). AC10 cardiomyocytes were plated at 30,000 cells/well and maintained in plateau phase of growth for 24 hours prior to doxorubicin exposure (represented by time = 0 hours). To reflect the clinical exposure parameters of doxorubicin against cardiac tissue, exposures were adapted to mirror the pharmacokinetic half-life of anthracycline of between 20 and 30 hours, mirroring doxorubicin pharmacokinetic exposure and drug metabolism within oncology patients (Eksborg, 1990, Barpe et al., 2010). AC10-CMs were exposed to the same initial concentrations of doxorubicin as per section 2.7.1, with the concentration of doxorubicin reduced by 50% every 24 hours to reproduce *in vivo* cardiomyocyte exposure to clinically relevant concentrations of doxorubicin.

From the time of drug administration, the plateau phase of growth was maintained for the first 6 hours (**Figure 3.6**), before increases in cell index were observed after 12 hours of continuous drug exposure. The increases in cell index were maintained until 24 hours after drug administration, most notably in the 500nM treatment group with a 5.3% increase in cell index recorded relative to control ($p=0.342$). Small, non-statistically significant increase in cell index were observed across the 250nM (4.0%, $p=0.341$), 100nM (2.7%, $p=0.543$), and 50nM (1.3%, $p=0.398$) doses relative to control, confirming lack of cytotoxic response at these concentrations, and indicating the dose-response relationship in relation to cardiomyocyte viability.

Following 24 hours of doxorubicin exposure (**Figure 3.6**), reductions in impedance measurements and therefore cell index were detected, especially at the higher doxorubicin concentrations of 250nM and 500nM, indicating cytotoxicity. The reductions in impedance measurements were observed until 72 hours, which represents the biological equivalent of 3 pharmacokinetic half-lives and is reflective of the systemic drug clearance of doxorubicin in the clinic. By 72 hours, AC10 cardiomyocytes exposed to 500nM doxorubicin were shown to have a 47.5%, statistically significant, reduction in cell index relative to control ($p<0.05$). Whilst reductions in cell index were also demonstrated within the 250nM (24.9%, $p=0.273$), 100nM (15.5%, $p=0.406$), and 50nM (20.9%, $p=1.61$) doxorubicin-exposed cardiomyocytes.

Despite sequential reductions in the doxorubicin concentrations, the cytotoxic effects of doxorubicin still predominated at high concentrations, albeit these concentrations were lower than the clinical Cmax of doxorubicin. The initial increases in cell index across all doses (50-500nM) relative to drug vehicle control (**Figure 3.6**) are unlikely to be related to increased proliferation because of the confluent nature of the culture, with a change in cellular morphology such as cellular hypertrophy the likely response. This response is in agreement with previous studies of AC10-CM with doxorubicin (Rockley, 2018).

In addition to demonstrating dose responses, these data also indicate the time-dependent nature of the cytotoxic response in quiescent/low-proliferation index cardiomyocytes. The lack of observed cytotoxic responses until after 24-hour exposures illustrates the requirement for latter time points as optimal for subsequent analyses of molecular changes, especially in quiescent as opposed to proliferative cardiomyocytes.

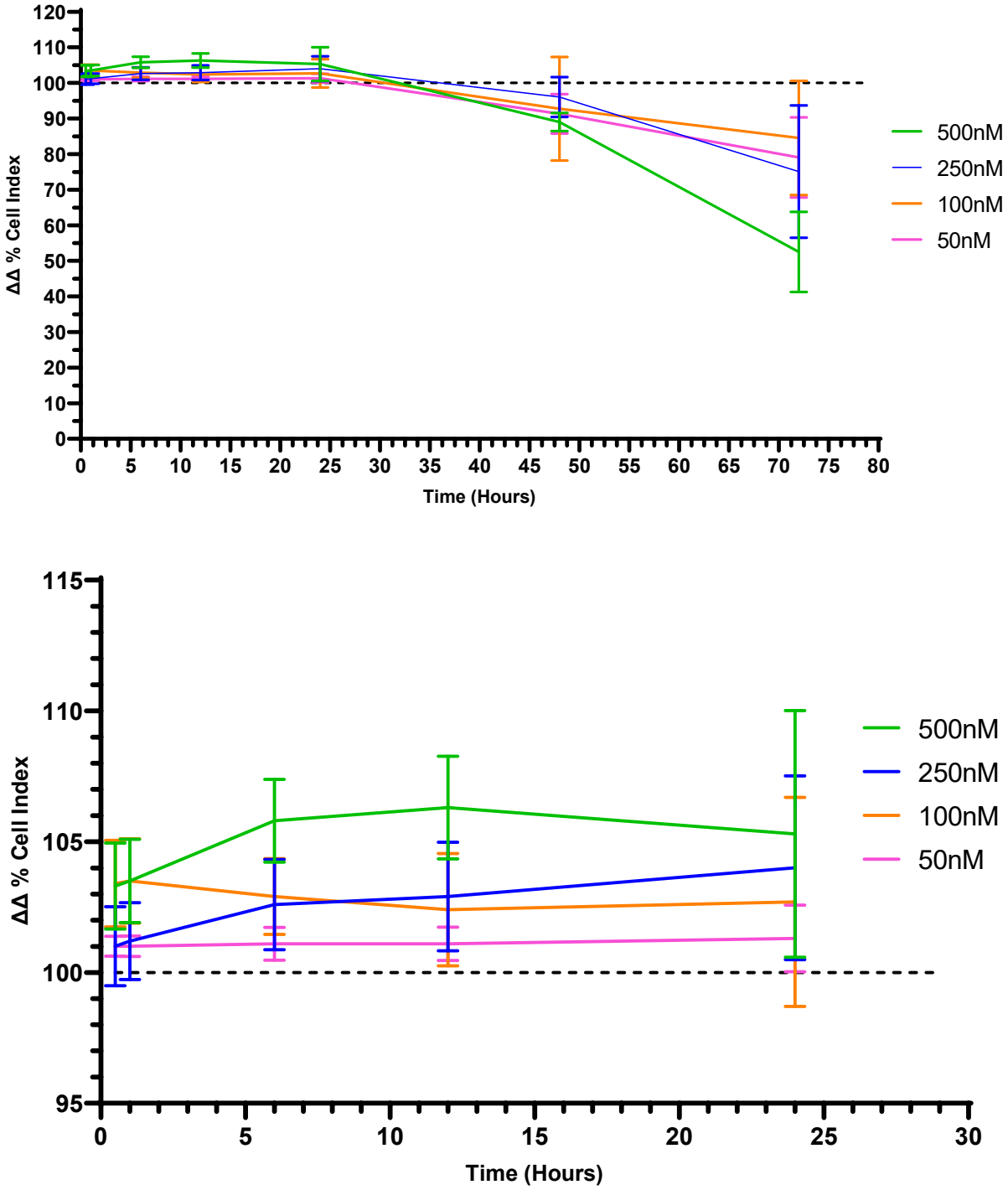


Figure 3.6 Doxorubicin induces a transient increase in cell index in AC10 cells before a decrease in cell index is observed. AC10 cells were exposed to doxorubicin for 72 hours. Following doxorubicin exposure, a transient increase in cell index, especially at the higher doses (250nM and 500nM) were observed. Impedance data are normalised to time of drug administration (baseline), then to time-matched vehicle control (ΔΔ% cell index) and are the average ±S.E. of at least 3 different experiments. Statistical analysis with paired t-test to compare each doxorubicin concentration to control.

3.7.7 Response of primary neonatal rodent cardiomyocytes in vitro to clinically relevant concentrations of doxorubicin, analysed in realtime by xCELLigence.

Primary neonatal rodent cardiomyocytes were cultured on E-plates to plateau (see section 2.6), and then exposed to the clinically relevant doses of doxorubicin (50, 100, 250 and 500nM), maintaining these respective drug concentrations throughout the duration of the study.

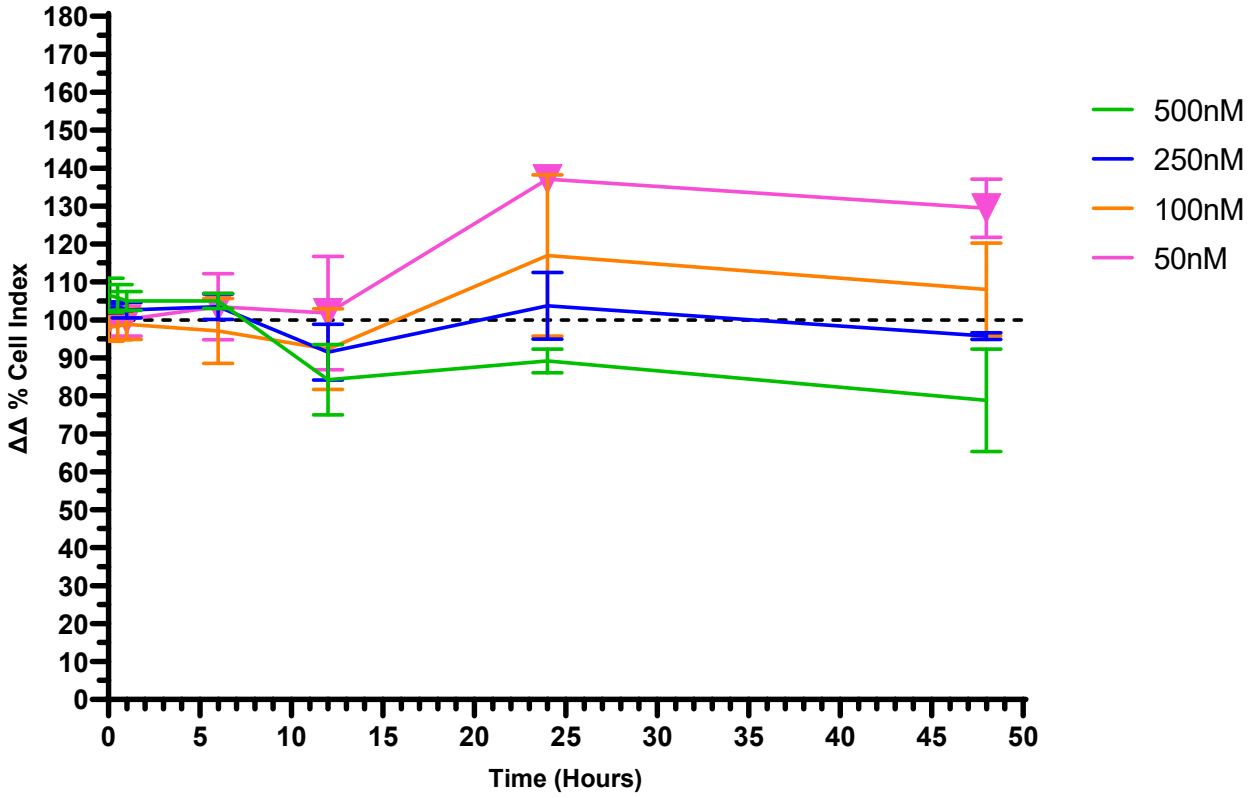


Figure 3.7 **Doxorubicin induces a transient increase in cell index in primary neonatal cardiomyocytes** before a decrease in cell index is observed. Primary cardiomyocytes were treated with varying doses of doxorubicin for 48 hours. Within the first 24 hours of doxorubicin expsoure, an increase in doxorubicin was observed, especially at doses 50nM and 100nM. A decrease in cell index was then observed from 24 hours. Impedance data has been normalised from time of drug addition, then to time-matched vehicle control (ΔΔ% cell index). Results are an average of at least 3 different experiments (S.E.), with paired t-test applied to evaluate for statistical significance.

For the highest doses of doxorubicin 250nM and 500nM, non-statistically significant reductions in cell index were observed within the first 12 hours post-doxorubicin exposure (**Figure 3.7**). Despite a transient increase in cell index up to 24 hours, the decline in cell index continued until the end of the experiment at 48 hours. The greatest increases in cell index at

24 hours were observed with the lower doxorubicin concentrations, with a 37.1% increase in cell index when cardiomyocytes were treated with 50nM ($p<0.05$); and a 17% increased at 100nM ($p=0.571$). These increases in cell index relative to control were maintained until 48 hours of doxorubicin exposure.

By contrast, following treatment with the higher doses 500nM and 250nM of doxorubicin for 48 hours, the greatest reductions in cell index relative to control were identified. Primary neonatal rodent cardiomyocytes exposed to 500nM demonstrated a 21.8% reduction in cell index compared to control ($p=0.362$), and a 4.2% reduction ($p=0.134$) at doses of 250nM doxorubicin.

3.7.8 Cellular response of primary neonatal rodent cardiomyocytes in the presence of decreasing exposures to doxorubicin to reflect pharmacokinetic profile

The effect of clinically relevant concentrations of doxorubicin upon the morphology and cellular survival of primary neonatal rodent cardiomyocytes were evaluated using xCELLigence real-time cellular analysis. The influence of sequential doxorubicin half-life treatments upon primary neonatal rodent cardiomyocytes was evaluated, using the previously determined clinical doxorubicin concentrations of 50, 100, 250, and 500nM. To replicate sequential in vivo drug pharmacokinetic half-lives, every 24 hours, 100µL of the doxorubicin-containing media was exchanged for 100µL of fresh media, thereby reducing the doxorubicin concentration by half each time, for the duration of the experiments.

The results are presented in **Figure 3.8** demonstrates that doxorubicin treatment promotes an increase in cell index within primary neonatal rodent ventricular cardiomyocytes. These effects become noticeable as early as 12 hours following doxorubicin exposure, continuing through to 72 hours. The greatest increases in cell index were observed in primary neonatal cardiomyocytes treated with 500nM doxorubicin, with 33.0% ($p=0.311$) and 102.2% ($p=0.129$) increases in cell index detected relative to control at 48 hours and 72 hours respectively. By 72 hours, increases in cell index following 250nM (86.6%, $p=0.435$), 100nM (80.4%, p0.539), and 50nM (18.7%, $p=0.745$) were also observed, though these did not reach statistical significance.

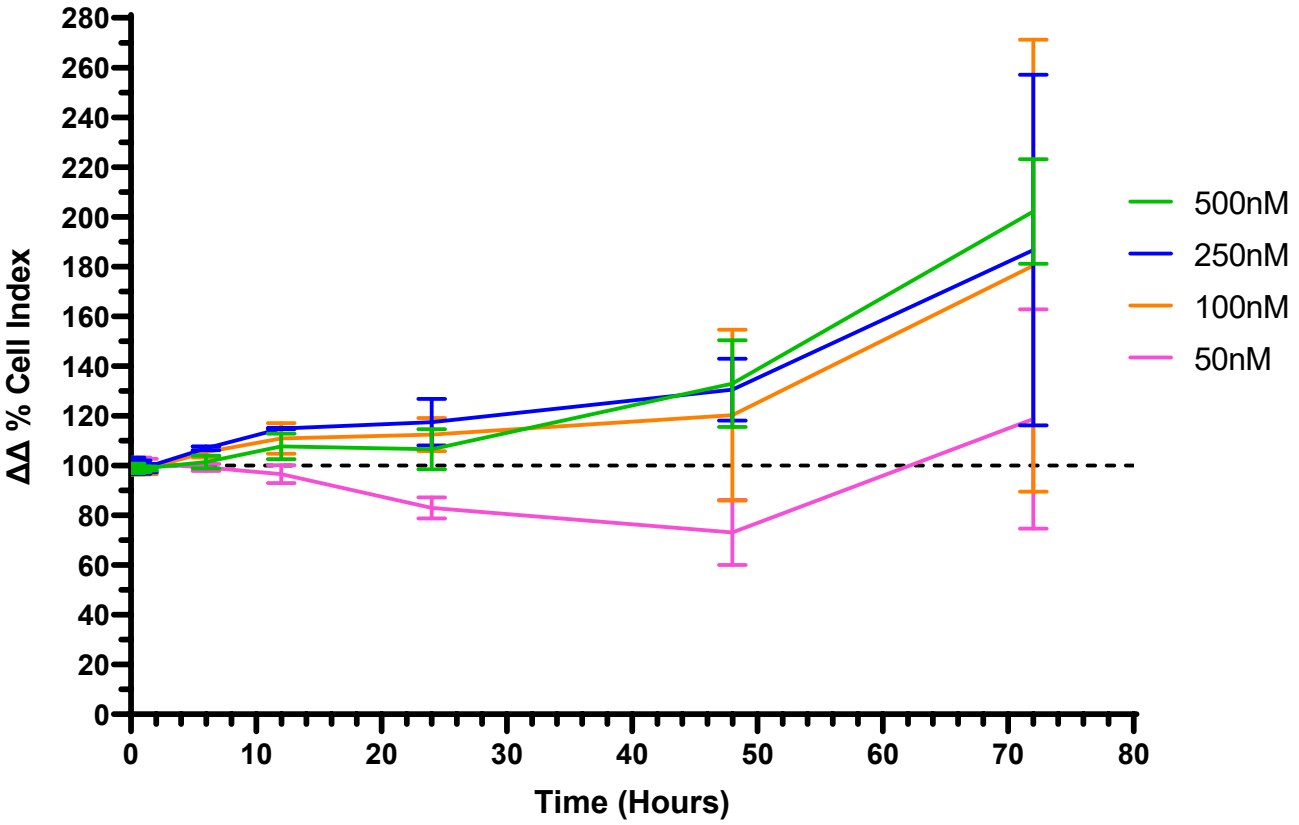


Figure 3.8 **Doxorubicin induces an increase in cell index in primary neonatal cardiomyocytes exposed to sequential half-lives**. Primary cardiomyocytes were treated with varying doses of doxorubicin for 72 hours, with media changes every 24 hours to reflect sequential biological half-lives. Up to 72 hours, increases in cell index across all doses (50-500nM) of doxorubicin were measured. The impedance data was normalised to baseline (i.e. time from drug addition), then to time-matched vehicle control (control (ΔΔ% cell index). Results are an average of at least 3 different experiments (S.E.), with the paired t-test used to evaluate statistical significance.

3.8 Pathological changes associated with anthracycline-induced cardiotoxicity in adult versus paediatric cardiac tissue

Cardiac tissue from patients exhibiting late-onset cardiotoxicity, received either from autopsy or at cardiac transplantation, had previously demonstrated the presence of interstitial and replacement fibrosis without a concomitant presence of significant necrosis, myocytolysis or cardiac hypertrophy (Bernaba et al., 2010). To address whether similar pathological changes are observed in cardiac tissue of paediatric cancer patients exhibiting late-onset cardiotoxicity, pathological reports and associated tissue histology were assessed retrospectively from cardiac tissue obtained at cardiac transplantation. Observations of ultrastructural and adaptive histological changes were compared to those from the previous study in tissue from adult patients (Bernaba et al., 2010).

Table 3.1 Heart weights and demographic information for paediatric patients

Patient	Sex	Age (years) at time of chemotherapy	Heart Weight (g)	Maximum normal heart weight for height (g)	Ratio of actual versus normalised heart weight
1	F	6.9	260	204	1.27
2	M	0.2	170	277	0.61
3	F	1.8	130	206	0.63
4	M	3.5	260	239	1.09
5	M	1.2	210	260	0.81
6	F	2.5	170	244	0.70
7	F	0.8	190	224	0.85
8	F	1.2	148	169	0.88
9	F	14.8	150	253	0.59
10	M	8.8	208	330	0.63
11	M	1.4	86	165	0.52
12	F	1.3	123	131	0.94
13	M	10.4	270	331	0.82
14	F	7.1	210	253	0.83
15	F	n/a	255	254	1.00
16	F	3.4	197	263	0.75
Mean (+/- SD)	**-**	**4.4 years ± 4.3**	**189.8 ± 54.6**	**236.4 ± 52.3**	**0.7883 ± 0.1055**

In the paediatric patient cohort, the average human heart ranged from 86-270 grams (mean = 189.8 ± 54.6) (**Table 3.1**). Calculation of the ratio of the actual patient's heart weight to the normalised heart weight (based on patient's height) was determined as 0.79 ± 0.11 (p=0.0014) (**Table 3.1**), indicating an overall 21% reduction in heart weight following exposure to anthracycline chemotherapy. This compares to an average heart weight of 317.5 ± 65 g and a ratio of actual to normalised heart weight of 1.1 ± 0.3 (p=0.22) in the adult heart following exposure to anthracycline chemotherapy (Bernaba et al., 2010)**.** This indicates the absence of cardiac hypertrophy in the paediatric cases, which agrees with the lack of hypertrophy detected in the adult hearts. In terms of ventricular wall thickness, the mean left and right wall thickness in the paediatric cancer patients with anthracycline-induced cardiotoxicity were recorded as 1.0 ± 0.2 cm and 0.5 ± 0.1 cm respectively. These measurements were similar to the adult cases (Bernaba et al., 2010), where the mean LV wall thickness was 0.5 ± 0.2 cm (p=0.0832) and RV wall thickness 0.5 ± 0.2 cm (p>0.99).

In contrast to the histological samples from adult cases of anthracycline-induced cardiotoxicity, which had no evidence of myocytolysis, 5 out of 16 (31%) histological sections from the paediatric cancer patients did show evidence of myocytolysis (**Figure 3.9**) (**Table 3.2**). In agreement with the adult cancer cases, all of the paediatric cancer patients exhibited interstitial fibrosis (**Figure 3.10**), with the majority demonstrating multi-focal fibrosis (**Table 3.2**). However, a greater proportion of the adult patients demonstrated diffuse (grade 3) fibrosis compared with the paediatric cases (30% versus 16%), whilst the paediatric patients displayed a greater degree of focal interstitial fibrosis (26% versus 10%) (**Table 3.2**). In terms of replacement fibrosis (**Figure 3.10**), this was similar between adult and paediatric cases of anthracycline-induced cardiotoxicity at around 60% of patients (**Table 3.2**). The sub-distribution of fibrosis within the paediatric cases also demonstrated a similar trend, with a greater proportion exhibiting focal (grade 1) relative to diffuse (grade 3) replacement fibrosis (**Table 3.2**). One interesting observation noted in the histological sections from the paediatric cancer cases was the presence of sub-epicardial foci for both interstitial and replacement fibrosis, an observation not reported for the adult patient population (Bernaba et al., 2010).

Table 3.2 **Cardiac histological observations of paediatric patients with late-onset anthracycline-induced cardiotoxicity**. Cardiac tissue, obtained from paraffin-wax embedded specimens produced following cardiac transplant, were sectioned and stained with haemotoxylin and eosin (H&E) and analysed microscopically for histological changes. Observations were compared to those previously reported by Bernaba et al. (Bernaba et al., 2010)

Patient Characteristics	Paediatric Tissue (n=16)	Adult Tissue (Bernaba et al.) (n=10)
Mean age at time of tissue retrieval (Years)	15 years	45 years
Patient Sex (Male/Female)	6/10	5/5
Mean duration between treatment and development of cardiomyopathy (Years)	9 years	6.2
Histological manifestations		
Necrosis Number of positive cases (%)	0 (0%)	0 (0%)
Myocytolysis Number of positive cases (%)	5/16 (31.3%)	0 (0%)
Interstitial Fibrosis: - ***Focal*** - ***Multi-focal*** - ***Diffuse***	16/16 (100%) 5/16 (26.3%) 11/16 (57.9%) 3/16 (15.8%)	10 (100%) 1/10 (10%) 6/10 (60%) 3/10 (30%)
Replacement Fibrosis: - ***Focal*** - ***Multi-focal*** - ***Diffuse***	9/16 (56.3%) 6/9 (66.7%) 2/9 (22.2%) 1/9 (11.1%)	6/10 (60%) 4/10 (40%) 2/10 (20%) 0/10 (0%)
Additional Features	Sub-epicardial foci	-
Mean Heart Weight (grams)	189.8	317.5
Mean LV thickness (cm)	1.0	1.2
Mean RV thickness (cm)	0.5	0.5

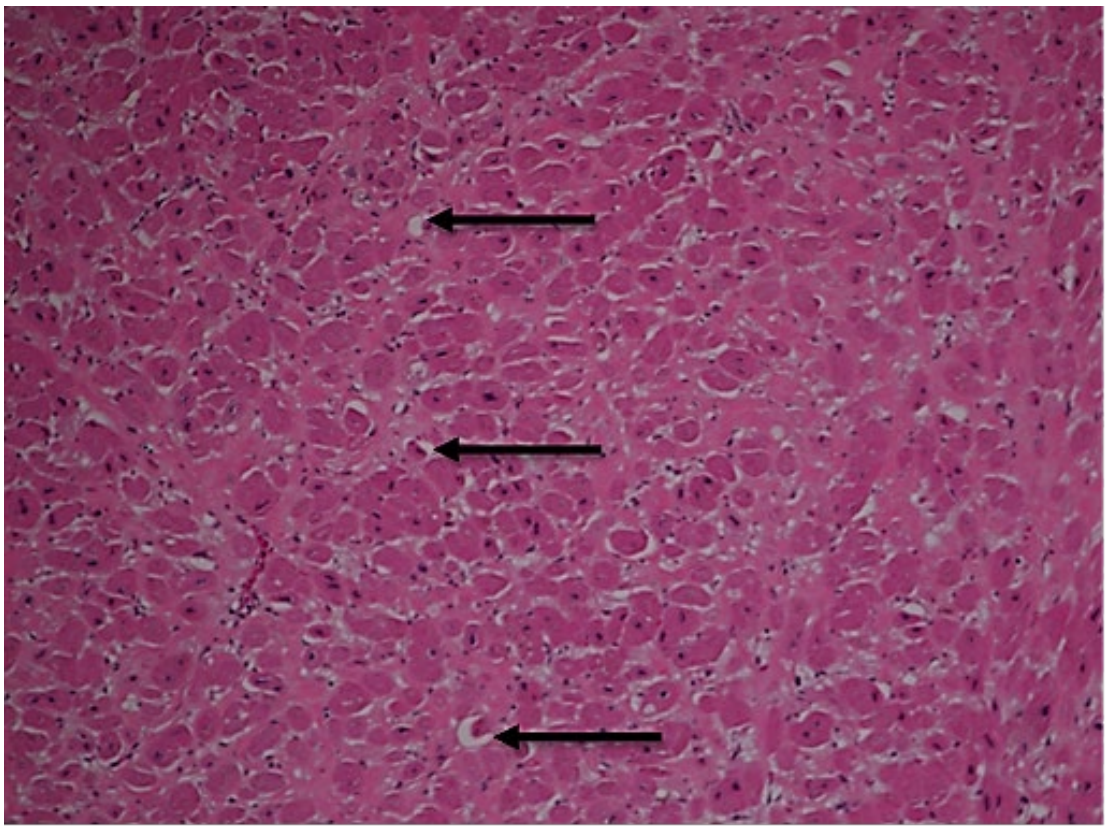


Figure 3.9 Histology of the left ventricle demonstrating myocytolysis (indicated by arrows)

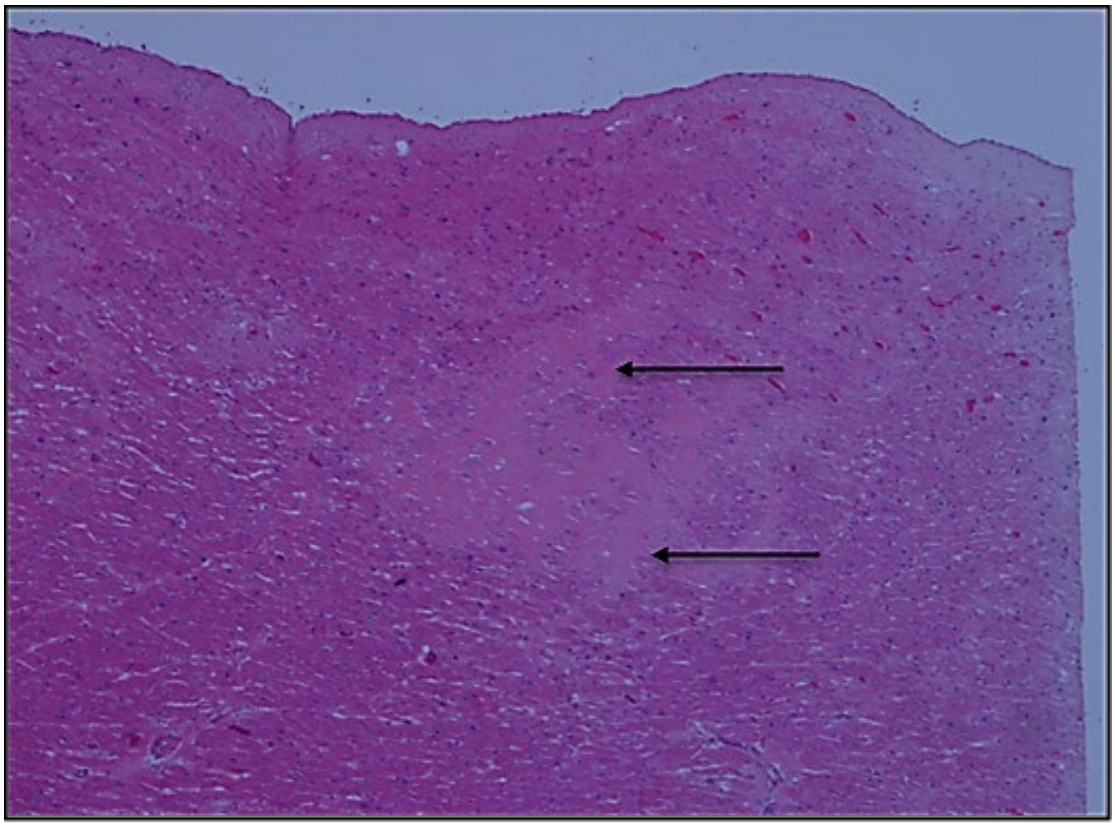


Figure 3.10 Histology of the left ventricle detailing interstitial fibrosis (indicated by arrows)

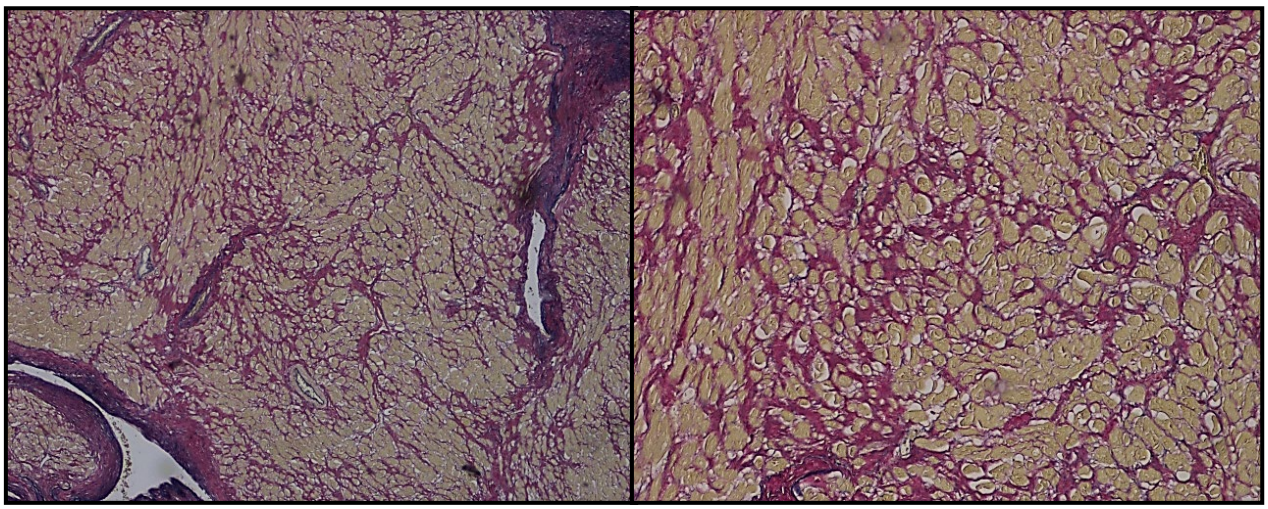


Figure 3.11 Histology of the left ventricle demonstrating replacement fibrosis

3.9 Discussion

The development of anthracycline-induced cardiotoxicity is now an accepted clinical adverse effect associated with these therapeutics in the management of cancer, presenting most often as a late-onset and chronic toxicity (Lotrionte et al., 2013, Lyon et al., 2022). Paediatric and adult patients are both vulnerable to myocardial dysfunction from anthracyclines for separate reasons. Children and adolescent patients exposed to toxic anthracycline doses are at high risk of permanently losing myocardial cells, especially as the cardiac tissue expands and develops. Although elevations in cardiac troponins are detectable in paediatric patients following anthracycline chemotherapy exposure (Michel et al., 2020b), indicating cardiomyocyte losses, it is the inhibition of cell expansion and development which is of greatest concern. Clinical studies evaluating cardiac structural changes in paediatric cancer survivors exposed to anthracyclines revealed progressive reductions in LV mass and cavity size, with serial echocardiograms demonstrating a decrease in patient's LV dimensions and wall thicknesses (Lipshultz et al., 1991, Lipshultz et al., 2014). To explain these crucial findings from a cellular perspective, De Angelis et al. (De Angelis et al., 2016), has demonstrated that losses of cardiac progenitor cells (CPCs) incurred by anthracycline chemotherapy may not only prevent full cardiac maturation, but also severely restrict the regenerative capacity of the heart, with subsequent adverse remodelling and fibrosis (Olivetti et al., 1991, Piegari et al., 2013, De Angelis et al., 2016). In cases of relatively low doses of anthracycline chemotherapy, CPC numbers and function may remain preserved. However, with repeated anthracycline exposures, greater CPC losses are expected which interferes with cardiac growth factor systems. Consequently, reductions in cardiac tissue structure and functional capacity ensue,

as survivors of childhood cancer previously exposed to anthracyclines are shown to develop accelerated myocardial dysfunction in contrast to healthy, non-exposed control patients (Olivetti et al., 1991, Lipshultz et al., 2014, De Angelis et al., 2016).

The pathway in adult patients differs in that the myocardium has already achieved full maturation, with CPC numbers already in decline as a natural consequence of ageing (Bergmann et al., 2015). In this context, if significant myocyte losses are incurred due to anthracycline exposure, a reduction in ventricular function is more likely to manifest, especially as the ageing cells possess a limited capacity for mitosis and regeneration of the tissue structure. To maintain a normal myocardial function and preserve cardiac output, the heart is reliant upon functional compensatory mechanisms, involving neurohormonal activation, sympathetic stimulation and mechanical adaptations, which are triggered in response to cardiomyocyte injury.

However, despite an appreciation of the pathological and physiological aspects of cardiotoxicity, the underlying molecular mechanisms and their relationship to the clinical manifestations of AIC is still unclear. The relationship between acute exposures to anthracyclines and delayed nature of the chronic cardiotoxicity remains unresolved. Determining this has vital clinical implications, as cardiotoxicity can remain undetected for many years after the completion of anthracycline chemotherapy, often presenting as symptomatic heart failure, for which prognosis is considered poor. Better understanding of the molecular mechanisms underpinning both acute and late-onset cardiotoxicity are required for better prognosis and management of this toxicity within the clinic. To achieve this objective, the first challenge addressed in this phase of the project was to establish an *in vitro* cardiac cell model, which can reflect both the cellular situation and responses to anthracyclines observed in the clinic.

The immortalised human AC10 ventricular cardiomyocyte cell line (AC10-CM), originally developed by Davidson et al. (Davidson et al., 2005), has previously been characterised and its suitability for *in vitro* studies of drug effects upon cardiomyocytes demonstrated (Davidson et al., 2005, Rockley, 2018, De Santis, 2020). Although confirmed as being in a pre-contractile state and devoid of sarcomeric structures, this cell line expresses many cardiac contractile proteins, including troponins, tropomyosin, and α-actinin; cardiac specific transcription factors GATA4, MYCD, NFATc4 and NKX2.5 involved in cardiogenesis and cardiac function; the marker

of cardiomyocyte differentiation BMP-2 and the presence of functional gap junctions exemplified by expression of Cx-40, Cx-43 (Davidson et al., 2005, Rockley, 2018). Additionally, the AC10-CM were also confirmed to exhibit proliferative potential, indicated by expression of pericentriolar material 1 (PCM1) and Ki-67 (Davidson et al., 2005, Rockley, 2018). The previously reported mesenchymal expression marker vimentin in AC10-CM, which is not expressed in cardiac cells, reflects the fact that AC10-CM are developed as a fusion between human cardiomyocytes and mitochondria-devoid human fibroblasts (Davidson et al., 2005). This represents a strength rather than a negative, as the myocardium is composed of cardiomyocytes alongside fibroblasts, the latter of which are responsible for a plethora of events in the myocardium including cardiac remodelling and cellular matrix deposition (Camelliti et al., 2005). These key properties therefore enhance the analysis of drug-induced morphological changes in response to the cardiac cellular environment. Taken together, these observations strongly support the qualification of AC10-CM as a human cardiac cell line suitable for *in vitro* studies of anthracycline-induced cardiotoxicity.

A broad range of pre-clinical cell-line models exist to evaluate the pharmacokinetic, cytotoxic, and morphological responses to anthracyclines. Neonatal rodent ventricular cardiomyocytes (NRVC) have previous been well characterised and applied as pre-clinical models for evaluating the pathophysiology of AIC (Dorr et al., 1988). NRVC were therefore selected to provide comparable analyses for the AC10-CMs, establishing the responses of ventricular cardiomyocytes to short-term doxorubicin exposure. Within the early neonatal stages, rat cardiomyocytes lose their capacity for mitogenesis, meaning total cardiomyocyte numbers remaining static. With cardiomyocyte terminal differentiation, myocardial development is dependent upon cellular hypertrophy and expansion of the extracellular matrix (Sen et al., 1988). While the myocardium is composed of multiple cell types, including, fibroblasts, endothelial cells and cardiac progenitor cells, specifically determining the pathological responses of the cardiomyocytes when exposed to different dose intensities and duration of anthracyclines is essential to understanding the mechanisms of cardiotoxicity. The protocol developed in section 2.6 and Appendix A, details the isolation of cardiomyocyte from the proliferating non-myocyte cells (e.g. fibroblasts) and extracellular matrix to produce a single cell culture. A considerable advantage of the primary neonatal rodent cardiomyocyte cell-line is the ability to synthesise a high yield of homogenous cells (i.e. cardiomyocytes), thereby promoting highly focussed *in vitro* analyses. Isolation of the cardiomyocytes enhances translatability into humans, as the cardiomyocytes are removed from extraneous stimuli and

hormonal influences delivered by the systemic circulation, which are known to influence growth kinetics and phenotypic properties. Furthermore, preliminary experiments applied to develop this protocol (section 2.6, Appendix A), identified the optimal *in vitro* conditions for NRVCs to undergo a dedifferentiation to re-differentiation process. Therefore, the cardiomyocytes are shown to regain their contractile phenotype *in vitro*. Although not utilised in this series of experiments, these properties can enhance *in vitro* evaluation through measuring chronotropic and inotropic responses, with spontaneous contractility maintained for at least 14 days (Nguyen et al., 2012).

Concentration and time-dependent responses of AC10-CM to doxorubicin were established, with corresponding IC_{50} cytotoxicity parameters calculated to gain a baseline appreciation of cellular responses. Cardiomyocytes are known to possess limited capacity for regeneration, so to recapitulate the adult human heart and clinical situation, wherein cardiomyocytes exhibit low proliferative capacity and quiescence, studies were conducted with both proliferative AC10-CMs and those grown to confluence. This equated to seeding densities of 2000 cells/well with drug exposure before day 3 post-seeding for exponential growth phase studies, and at day 5 post-seeding for studies in the plateau growth phase, for investigations utilising a 96-well culture plate format. In terms of the real-time impedance-based assays (xCELLigence), AC10-CMs analyses appraised cells seeded at 30,000 cells/well and analysed at 24 or 96 hrs to attain exponential and plateau phases of growth, accordingly.

Doxorubicin is the most frequently used and archetypal anthracycline, notoriously associated with cardiotoxicity (Valcovici et al., 2016). Acute doxorubicin exposure produced a dose-dependent reduction in cardiomyocyte viability in both proliferative and quiescent cell models. Interestingly, proliferative cells exhibited a lower cytotoxic response to doxorubicin relative to quiescent cells with low proliferative rates, with IC_{50} values of 1.55µM (C.I. 1.2 to 2.1µM) and 182.5nM (C.I. 101 to 301nM) after 24 hours, respectively, as determined by the MTT cell viability assay. A similar differential response was observed using the alternate trypan blue exclusion assay, with IC_{50} values of 1.1µM (C.I. 0.5 to 2.3µM) and 486nM (C.I 317 to 748nM), respectively. The differences in IC_{50} values derived from the MTT and trypan blue assays can be explained by the specific mechanism used to determine cell viability. The trypan blue methodology utilises the integrity of the cell membrane as a marker of cell viability i.e. compromised cell membranes all uptake of the trypan blue dye, which binds to intracellular proteins. This evaluation of the compromised versus non-compromised cell membranes

provides a marker for cell viability. By contrast, MTT assays determines cell viability by measuring intracellular activity, as dye uptake is mediated by active transport and incorporated into viable cell lysosomes. Reductions in cellular metabolism and lysosomal damage both decrease cellular dye accumulation, acting as a surrogate for cell viability (Masson-Meyers et al., 2016). When combined, these two assays yield valuable data regarding the biochemical (intracellular uptake and metabolism) and physical (cell membrane integrity) responses of the cardiomyocytes to doxorubicin, with the results from the IC_{50} analyses indicating that both markers of cell viability are adversely affected by doxorubicin, with quiescent cardiomyocytes more susceptible to doxorubicin compared with actively proliferating cells.

Although these cytotoxicity values within the quiescent state are considerably less than the Cmax of doxorubicin in humans (1.1µM), they are however clinically relevant, as following administration doxorubicin undergoes a rapid distributive phase of 3 to 5 minutes, before a terminal half-life phase of 20 to 30 hours (Eksborg, 1990, Barpe et al., 2010). As such, the concentration of active drug that will be exposed to cardiac tissue will be subsequently lower than the Cmax due to doxorubicin metabolism and distribution within the tissues. Previous studies by Rockley using AC10-CMs reported the doxorubicin Cmax as 1.1µM (Rockley, 2018). However, the calculated IC_{50} values of 14.7nM for proliferating cardiomyocytes, and 29nM for quiescent cardiomyocytes, which contrasts with the results detailed in **Figure 3.4** and **Figure 3.5**. A proposed explanation for these differences within the existing literature derives from the specific methodologies applied, with the IC_{50} values in Rockley (2018) exposing cells to doxorubicin for 24 hours, before allowing a 72-hour recovery period before cell viability analysis. Further analyses from Rockley (2018) exposed AC10 cardiomyocytes to doxorubicin continuously for 96 hours, before completing the cell viability analysis, with these IC_{50} values similar to the 24 hours exposure plus 72 hours recovery (Rockley, 2018). These results indicate that the cardiotoxic effects of doxorubicin are not immediate, and that an observed lag period of at least 24 hours is required before significant reductions in cell viability are detectable. This hypothesis is supported with analyses from Chularojmontri et al., where the recorded IC_{50} value of 142nM following 48 hours exposure to doxorubicin in the rat H9c2 cardiomyoblast (Chularojmontri et al., 2013). Together these findings indicate the progressive nature of doxorubicin cardiotoxicity, with cardiomyocytes initially exposed to doxorubicin highly susceptible to cellular damage and cell death. This suggests that through prolonged doxorubicin exposure, for example patients known to have an elevated blood plasma

anthracycline concentration (i.e. elderly patients) (Findlay et al., 2019), or patients with repeated doxorubicin doses are vulnerable to the aforementioned cardiotoxic effects, especially due to the insufficient myocardial recovery time between treatment cycles.

A recognised challenge within the oncology clinics is how to maximise the therapeutic benefit of the anthracycline chemotherapy, whilst simultaneously negating their adverse cardiac effects. Anthracyclines are limited by dose-related cardiotoxicity, with patients receiving cumulative doxorubicin doses ≥ 250mg/m^2 considered at a higher risk, and potentially requiring alternative cancer treatment strategies (von Hoff et al., 1979, Lyon et al., 2022). In addition to restricting the total cumulative dose, the delivery of anthracyclines has been explored, with infusion durations modified and novel formulations developed to optimise the safety and efficacy of anthracycline chemotherapy. Previous studies have indicated that doxorubicin concentrations at or near to the Cmax (maximum serum concentration) are strongly associated with both histological and clinical signs of cardiotoxicity (Legha et al., 1982, Ishisaka et al., 2006). To closely reflect the doxorubicin pharmacodynamics in patients with cancer, careful consideration was given to the doxorubicin dosing and drug exposure schedules within *in vitro* experiments. As the doxorubicin Cmax levels are typically reached either during or shortly after infusion, AC10 cardiomyocytes and primary neonatal cardiomyocytes were treated with doxorubicin close to and below the known Cmax, with the cardiomyocytes analysed for acute cardiotoxic effects in the following hours. These experiments (**Figure 3.6** and **Figure 3.7**) demonstrated that cardiomyocytes exposed to the highest doxorubicin doses (500nM and 250nM) sustained cardiomyocyte losses to indicate cardiotoxicity (as represented by reductions in cell index), despite these doses being below the previously calculated Cmax.

As doxorubicin Cmax is both dose- and schedule-dependent, important clinical trials have been completed to evaluate both the cardiotoxic and anti-tumour effects derived from lowering the peak doxorubicin concentration. Studies targeting the doxorubicin infusion duration, consistently found that bolus administration (5 minutes) was associated with higher rates of both clinical and histologically-defined cardiotoxicity, when compared with prolonged infusions (24-96 hours) (Legha et al., 1982, Bielack et al., 1989, Shapira et al., 1990). These shorter infusion rates were associated with up to a ten-fold increase in mean plasma doxorubicin levels, compared with the slower infusion rates, with subsequent meta-analysis data revealing a 4-fold higher risk of clinical cardiotoxicity associated with the bolus infusions (Legha et al., 1982, Smith et al., 2010). Therefore, these findings demonstrate that

cardiotoxicity could be related to peak plasma levels of the drug (Cmax), and prolongation of the doxorubicin infusion may even be cardioprotective. To ensure these continuous doxorubicin infusions could be safely administered to patients in the clinic, the anti-tumour effects were also evaluated. Non-randomised studies have demonstrated comparable anti-tumour efficacy between the bolus and continuous infusion regimens, indicating that the therapeutic efficacy is related to the total doxorubicin exposure rather than the Cmax (Legha et al., 1982, Bielack et al., 1989, Shapira et al., 1990). The total doxorubicin exposure was quantified by measuring the plasma doxorubicin concentration across time, with these values represented as 'area under the concentration-time curve' (AUC). Therefore, these studies established that doxorubicin Cmax could determine the likelihood of cardiotoxic effects, whilst the AUC was more closely, associated with the therapeutic benefit. Cmax does have some influence upon the anti-tumour efficacy, however, with Ishisaka et al. (2006) demonstrating changes in survival rates of in vitro leukaemic cells during different durations of doxorubicin exposure, after AUC values were standardised (Ishisaka et al., 2006).

To further enhance the safety profile of doxorubicin and maintain therapeutic efficacy, novel formulations encapsulating doxorubicin within small pegylated liposomes have been developed, designed to increase its circulatory half-life, whilst limiting peak doxorubicin exposure to the myocardium. A meta-analysis comparing liposomal-encapsulated doxorubicin to conventional doxorubicin found similar tumour response rates and progression-free survival, with the liposomal formulations producing a 38% relative risk reduction in heart failure development, again supporting the implementation of strategies to reduce doxorubicin Cmax (van Dalen et al., 2010). However, the increased costs of liposomal doxorubicin have limited its widespread use in clinical practice, with the recent ESC cardio-oncology guidelines reserving it for patients assessed as high and very high risk of developing cardiotoxicity or patients who have already received high cumulative anthracycline doses (Lyon et al., 2022).

The safe application of these infusion regimens and novel formulations is less clear in the paediatric patient setting, with a paucity of randomised trial evidence (van Dalen et al., 2016). Typically, paediatric anthracycline chemotherapy dosing is calculated according to body surface area or weight, however, this approach can produce large dosing discrepancies between patients of the same age (Sallustio and Boddy, 2021). These variations in anthracycline dose combined with differences in drug clearance within children, may not only increase the intensity of therapy, but expose the developing paediatric myocardium to the

risks of cardiotoxicity and limit long-term survival (Siebel et al., 2020). Further research studies are therefore imperative to firmly establish the role of pharmacokinetic predictors of doxorubicin responses (e.g. Cmax and AUC) within clinical practice, supporting dose optimisation to strengthen cancer therapeutics and promote anthracycline safety. These pharmacokinetic targets and predictive models have the potential to individualising anthracycline treatment, whilst successful integration with cardiac biomarkers and imaging would maximise anti-tumour efficacy and enhance cardioprotection.

The mechanisms for doxorubicin transport into cancer cells and their nuclei are well defined, and explain the physiological basis for the lack of cytotoxic response within the first 24 hours following treatment exposure (Dalmark and Storm, 1981, Lal et al., 2010). Doxorubicin is described as entering tumour cells by passive diffusion, and then translocated into the nucleus and mitochondria, ready to induce its cytotoxic actions (Kiyomiya et al., 2001). Due to its importance regarding drug pharmacokinetics, anthracycline transport has previously been studied in hepatic tissue. Small fenestrations within the liver sinusoidal endothelial cells facilitate low-pressure ultrafiltration of doxorubicin from the portal blood to the hepatocytes, priming the doxorubicin for hepatic metabolism prior to biliary excretion (Hilmer et al., 2004). Liposomal formulations of doxorubicin, which have shown reduced cardiovascular toxicity (Shapira et al., 1990, Crombag et al., 2016), appear unable to diffuse across the endothelial barrier, as demonstrated by a significantly reduced hepatic uptake compared to free doxorubicin, therefore impairing hepatic metabolism (Hilmer et al., 2004). In comparison, cellular kinetics of anthracycline uptake and handling in cardiac tissue has gained limited attention, with most pharmacokinetic studies assuming comparative processes to non-metabolic tissues and uptake driven passively based on drug lipophilicity (Dalmark and Storm, 1981, Frezard and Garnier-Suillerot, 1991, Aryal and Rao, 2016). However, given the ever-increasing significance of anthracycline-induced cardiotoxicity, greater understanding of events associated with initiation of anthracycline-induced cardiotoxicity, such as kinetics of cellular uptake and handling, is fundamental to improving understanding and management of this clinical issue. Although cardiac quantification of doxorubicin has been studied in rodents, there remain only a few studies appraising doxorubicin quantification and cellular kinetics in human cardiomyocytes (Luo et al., 2017, Wang et al., 2020, Huang et al., 2021). In the most recent study by Huang et al. (Huang et al., 2021), although cellular uptake mechanisms of doxorubicin were evaluated using human-induced pluripotent stem-cell derived

cardiomyocytes (hiPSC-CMs), this was not related to a quantification of doxorubicin intracellular levels (Huang et al., 2021).

In contrast to historical studies, which recognise passive diffusion as the mechanism for doxorubicin uptake into cardiac cells and intracellular retention, recent studies indicate that it may in fact be driven by a transporter-mediated mechanism, with several membrane proteins identified (Lal et al., 2010, Huang et al., 2021). In particular, the organic cation transporter 3 (OCT3) has been identified as a key player in this context, being the sole cationic transporter capable of driving cardiac uptake of doxorubicin (Huang et al., 2021). The role for OCT3 and its importance with regards doxorubicin-induced cardiac injury was further exemplified through use of selective inhibition of this transporter, which slowed uptake of doxorubicin into hiPSC-CMs *in vitro* and could preserve cardiovascular function *in vivo* without compromising the anti-tumour effects of the drug (Huang et al., 2021). Similarly, although blockade of OCT3 prevented uptake of doxorubicin into cardiac cells, it did not affect plasma levels of the drug *in vivo*, consistent with a lack of impact or involvement of this transporter in metabolism or excretion of doxorubicin (Huang et al., 2021). In studies using hiPSC-CMs, used to evidence the role of OCT3 in anthracycline-induced cardiotoxicity, the expression of OCT3 is reportedly highly upregulated in hiPSC-CMs obtained from patients experiencing cardiotoxicity relative to those that were not (Huang et al., 2021). This may suggest either drug-induced upregulation of OCT3 or more likely differential expression levels of OCT3 associated with susceptibility to cardiotoxicity. Recently, genetic polymorphic variants in OCT3 have been shown to contribute to degree of cardiac artery disease (Wang et al., 2016, Zhao and Zhang, 2017), implicating gain or loss of function of these transporters as potential drivers for susceptibility to conditions such as anthracycline-induced cardiac injury (Huang et al., 2021). It remains to be determined whether or not the uptake and retention of doxorubicin in the AC10-CM model *in vitro* is a direct consequence of OCT3 expression and activity, and crucially whether this closely reflects the clinical scenario.

Whilst the liver is the principal target for enzymatic reduction of doxorubicin, with anthracyclines contraindicated in patients with severe hepatic impairment, *in vitro* human cardiomyocytes have demonstrated an independent ability to convert doxorubicin to its active metabolites (Olson et al., 1988, Minotti et al., 1995). As the anthracycline metabolites (e.g. doxorubicinol, agylcones) have a greater polarity compared with their parent drug, this would promote doxorubicinol retention within the cardiomyocytes and impair elimination, thereby

prolonging cytotoxic exposure. Pharmacokinetic studies in both human and rodent cardiac tissues, have indicated prolonged cardiac retention of doxorubicin, and even the presence of doxorubicinol with cardiac tissues (Stewart et al., 1993, Lu et al., 2004). Fluorescence imaging has also revealed that upon entering cardiomyocytes, doxorubicin possesses a high affinity for the nucleus and mitochondria. This rapid organelle accumulation was shown to induce significant intracellular oxidative stress, even within the first 20 minutes of doxorubicin exposure, whilst prolonged doxorubicin incubation (20 hours) was found to enhance these acute effects (Sarvazyan, 1996). These studies demonstrate that in addition to the immediate and direct effects induced by doxorubicin, cardiomyocytes are vulnerable to prolonged cardiotoxic processes. Therefore, because of cumulative dose-related exposure, significant cardiomyocyte losses will be incurred, and even potentiate the reductions in cardiac mass observed in paediatric and adult patients (Lipshultz et al., 2005, Jordan et al., 2018). The cardiomyocyte accumulation of doxorubicinol, amplified by repeated doxorubicin dosing, may even explain the origin of chronic cardiotoxicity (Licata et al., 2000). Further studies are necessary to measure the concentrations of doxorubicin and its metabolites within cardiomyocytes to help explore the intracellular cardiotoxicity mechanisms.

Interpolation of studies using real-time impedance-based analyses (xCELLigence), evaluations of time-dependent doxorubicin exposure confirmed an unchanged cell index measured within the first 24 hours, following doxorubicin administration to AC10-CMs cardiomyocytes in culture and across all doses (50-500nM) (**Figure 3.6**). Following this initial period, doxorubicin cytotoxicity was observed across the range of clinically relevant concentrations, based upon known doxorubicin Cmax values (1.1µM) (Minotti et al., 2004), as evidenced by the reduction in cell index from 24 hours to experimental completion, 72 hours post-dose (i.e. 72 hours = biological equivalent of 3 half-lives). As previously discussed, this initial lag phase prior to the cytotoxic response likely reflects doxorubicin cardiomyocyte uptake, in addition to the time taken for disruption of intracellular signalling pathways with respect to induction of cellular stress and damage. In support of this, despite sequential reductions in doxorubicin concentrations against AC10-CMs, replicating *in vivo* cardiomyocyte exposure to doxorubicin through systemic drug clearance, the greatest decreases in cell index were recorded between 48 and 72 hours, with a 48% reduction in cell index ($p<0.05$) relative to control when doses of 500nM were administrated (**Figure 3.6**). Therefore, even though the drug was effectively being cleared from the media (by completely removing the doxorubicin-containing media, rinsing the cells, and administering fresh media), the cytotoxic effects of doxorubicin were still

being induced, as represented by a persistent reduction in cell index. This suggests that once the cytotoxic effects of doxorubicin begin, they may be limited by increases in drug metabolism and the sequential half-lives, however, the early high-dose exposure has a significant impact upon cardiomyocyte survival, and the effects produced within the first 24 hours exposure are not easily reversible.

Exposure of primary neonatal ventricular cardiomyocytes (PNVMs) to doxorubicin produced a transient increase in cell index within the first 12 hours, which was maintained until 24 hours following doxorubicin exposure, with cardiomyocytes exposed to the lower doses of 50nM and 100nM demonstrating an increase in cell index up to 37% (**Figure 3.7**) relative to vehicle control. Between 24 and 48 hours post-doxorubicin dosing, reductions up to 42% in cell index were identified, again indicating the induction of a cytotoxic response within the cardiomyocytes, notably at the highest doxorubicin concentrations (250nM and 500nM). When PNVMs were treated with doxorubicin, and sequential half-lives of the drug to model drug elimination over three successive half-lives (72-hours), overall increases in cell index relative to vehicle control were detected on the xCELLigence technology (**Figure 3.8**). From a physiological perspective, although the neonatal cardiomyocytes are known to have a low proliferation rate from birth, they are still within their developmental stages. Therefore, the increase in cell index is consistent with cardiomyocyte hypertrophy and formation of a functional syncytium within the PNVM model, ensuring the heart reaches its maximal volume and capacity to maintain sufficient cardiac output to meet the increasing systemic metabolic demands(Bergmann et al., 2015). Lewis et al. (1986), revealed that within just 24 hours of doxorubicin exposure, *in vitro* neonatal rat cardiomyocytes displayed dose-dependent ultrastructural changes, specifically cytoplasmic disorganisation and the depolymerisation of actin filaments fundamental to myocardial contractility (Lewis and Gonzalez, 1986). This cardiomyocyte disarray may also explain the key findings of Sag et al., who detected calcium leakage from the sarcoplasmic reticulum in wild type rat hearts treated with doxorubicin, with calcium losses contributing to myocardial dysfunction (Sag et al., 2011). Therefore, whilst the cardiomyocyte numbers may remain constant, physiologically significant ultrastructural changes within the cardiomyocytes and supporting architecture are the prime indicators of high degrees of cardiotoxicity.

Compounding the formation of reactive oxygen species and cardiomyocyte apoptosis, the inhibition of DNA damage responses and repair mechanisms, prevents any meaningful

myocyte regeneration, as evidenced within *in vivo* studies culminating in cardiac failure post-doxorubicin exposure (Zhang et al., 2012). Interestingly, Monti et al. (1986) demonstrated that single dose administration of doxorubicin in adult rat hearts causes a biphasic response in relation to myocardial contractility. As evidenced by an 'acute phase' of contractile impairment (up to one week) followed by a 'recovery phase', all within the first three weeks post-drug administration. A subsequent 'delayed cardiotoxicity response' was later observed at eight weeks post-anthracycline treatment, as evidenced by irreversible morphological and functional myocardial impairment (Monti et al., 1986). Although this study was exclusively in atrial cardiomyocytes, it strongly indicates a delayed cardiotoxic response, and that the changes caused by immediate compensatory mechanisms observed in **Figure 3.7** and **Figure 3.8** are insufficient to prevent manifestation of the cardiotoxicity phenotype. These studies have explored the acute cardiomyocyte-specific changes in response to doxorubicin administration. Comprehensive examination of the myocardial histology will not only identify the effects of doxorubicin upon other relevant cell types but may differentiate acute and chronic anthracycline-induced cardiotoxicity responses.

Analysis of hearts explanted prior to cardiac transplantation from paediatric patients diagnosed with chronic anthracycline-induced cardiotoxicity, revealed a statistically significant reduction (21% overall) in actual heart weights compared to the calculated normalised heart weights for these patients (**Table 3.1**). These key results are consistent with echocardiographic data recorded in childhood cancer survivors exposed to anthracycline chemotherapy, which determined long-term survivors to have a below normal left ventricular mass, wall thickness and contractility post-chemotherapy (Lipshultz et al., 2012). Lipshultz et al. coined the term 'Grinch syndrome', to describe these reductions in myocardial dimensions and cavity sizes relative to individual patient's body size (Lipshultz et al., 2014). Serial echo data from the same study indicates a progressive nature to the doxorubicin-induced cardiotoxicity, with initial echocardiograms representing a subclinical dilated cardiomyopathy, whilst echocardiograms beyond 15 years post-anthracycline exposure reveal a predominantly restrictive cardiomyopathy, supported by a relative decrease in left ventricular dimensions (Lipshultz et al., 2005, Lipshultz et al., 2014). Further studies applying cardiac MRI technologies to evaluate cancer patients for anthracycline-induced cardiomyopathy have also confirmed reductions in left ventricular mass and LVEF (Neilan et al., 2012, Jordan et al., 2018).

The direct comparison between the explanted heart weights of the paediatric patients in (**Table 3.1**) and the adult patients within Bernaba et al. (2010) indicates a difference in the explant heart weights. The paediatric study found 13 of the 16 patients to have a reduction in the heart weights relative to the expected (normalised) heart weights (ratio 0.79, p=0.0014). By contrast, histological analyses from Bernaba et al. (2010) measuring explanted hearts in adult patients diagnosed with anthracycline-induced cardiomyopathy, revealed no significant difference in normalised heart weights (ratio 1.1, p=0.22) (Bernaba et al., 2010). Considering myocardial development, these findings signify that anthracycline-induced cardiomyopathy in childhood cancer survivors causes suppression of myocardial growth, appearing to limit the growth potential of the myocardium, as evidenced by a reduced left ventricular mass in these patients. The lack of difference within the adult patients indicates preservation of cardiac mass following chemotherapy. Evaluation of these findings signals the potential for separate pathological responses and progression when comparing paediatric and adult populations with regards to anthracycline-induced cardiomyopathy. Based upon the available literature, the most plausible explanation for these differences is the relative stages of myocardial development and the composition myocardial cells affected at the time of anthracycline chemotherapy exposure (Bergmann et al., 2015).

To discover the underlying pathology of anthracycline-induced cardiomyopathy, a retrospective analysis of endomyocardial biopsy and autopsy samples by Steinherz et al. (1995), identified myocyte loss, increased hypertrophy and myocardial fibrosis associated with symptomatic late-onset cardiomyopathy in patients successfully treated with anthracycline chemotherapy as children (Steinherz et al., 1995). Although the detailed histological analysis informing **Table 3.2** presented isolated cases of cardiomyocyte hypertrophy within the paediatric cohort, this was not a consistent or predominant feature of the explanted hearts examined, and similarly not detected in the adult heart explants evaluated by Bernaba et al. (Bernaba et al., 2010). Integration of these observations and the available cardiac imaging data within the literature, crucially reveals that anthracycline-induced cardiomyopathy may in fact be a unique form of cardiomyopathy, deviating from the archetypal dilated, hypertrophic and restrictive cardiomyopathies classification, with potentially overlap of their specified features. The results from Steinherz et al. could indeed be explained by the limited of use of ACE inhibitors within the anthracycline-induced cardiomyopathy patients. These patients presented with symptomatic heart failure, late within the course of their cardiomyopathy progression (6 to 19 years post-anthracycline chemotherapy), with cardiac failure treatment

consisting of digoxin and furosemide (as key ACE inhibitor trial data emerged circa 1994) (Steinherz et al., 1995). During cardiomyopathy progression, increased left ventricular afterload and wall tension, known to be hypertrophy-inducing, could have been reduced/prevented with early ACE inhibitor therapy. Improvements leading towards modern heart failure guideline-directed therapy, may explain the lack of cardiomyocyte hypertrophy observed in **Table 3.2** and the Bernaba et al. (2010) publication (Bernaba et al., 2010), with early afterload reduction and impairment of the hypertrophic response, linked to the progression of late-onset anthracycline-induced cardiotoxicity.

In terms of cellular changes at the organ level and their relationship to cardiac function, analyses of explanted hearts from adult patients previously diagnosed with anthracycline-induced cardiotoxicity demonstrated significant grades of both interstitial and replacement fibrosis. The early reported cardiac histological features of anthracycline toxicity within the literature include myocardial inflammation, myocyte swelling and intra-myocyte vacuolisation (Bristow et al., 1978b). With subsequent anthracycline exposure, the cardiac damage has been shown to become more diffuse, promoting myocyte necrosis and permanent myocyte loss (Meinardi et al., 1999). Cardiac myocytolysis, characterised by myofibrillary loss and subendocardial vacuolisation between the myocardial fibres, typically develops as a result of myocyte necrosis and its histological presence is most frequently attributed to myocardial ischaemia. Furthermore, it is recognised as the intermediate stage between acute inflammatory cell activation combined with intramyocellular oedema, and diffuse chronic fibrosis in response to cardiomyocyte injury (Schlesinger and Reiner, 1955, Turillazzi et al., 2005). Due to the chronicity of the cardiotoxicity present in the evaluated paediatric patient cohort (section 3.8), inflammatory changes and necrosis were not identified within any of the histological samples analysed, whilst no ischaemic heart disease was recorded within any of the paediatric patients prior to cardiac explantation. Myocytolysis was however observed within almost 50% of the paediatric histological samples, and thus represents the first published cohort study in association with anthracycline-induced myocardial damage in survivors of childhood cancer (**Table 3.2**). Whilst Bernaba et al.'s analysis found no evidence of myocytolysis in adult cardiac transplant patients diagnosed with anthracycline-induced cardiotoxicity (Bernaba et al., 2010), there are subsequent histological studies demonstrating myocytolysis together with myocyte hypertrophy in adult patients treated with anthracycline chemotherapy (Cascales et al., 2013, Segura et al., 2015). Although no direct correlation has yet been found between the degree of myocytolysis and disease, these more recent adult

studies suggest the presence of myocytolysis may be related to subclinical myocardial dysfunction and even be a precursor for congestive cardiac failure (Cascales et al., 2013, Segura et al., 2015). The novel finding of myocytolysis and cardiomyocyte vacuolisation within the paediatric histological samples demonstrates that this phenomenon is not uniquely present in adults with established anthracycline-induced cardiotoxicity, but also paediatric survivors of cancer previously exposed to anthracyclines. Despite the presence of interstitial fibrosis in all the paediatric cohort study samples (**Table 3.2**), there was no correlation observed between myocytolysis and the grade of fibrosis. Whilst replacement fibrosis was seen 4 out of 5 (80%) of the paediatric cases with detectable myocytolysis, further large-scale histological studies in patients exposed to anthracycline chemotherapy are necessary to determine at which point in the clinical spectrum of anthracycline-induced cardiotoxicity that myocytolysis, or cardiomyocyte vacuolisation, becomes detectable and how this influences clinical prognosis.

The development of myocardial fibrosis, on the other hand, has previously been shown to manifest later within the course of anthracycline-induced cardiotoxicity and has the potential to become a marker for future myocardial dysfunction (Bernaba et al., 2010, Farhad et al., 2016). The histological analysis with the paediatric cohort (**Table 3.2**) confirmed the presence of ventricular remodelling in response to anthracycline-induced cardiotoxicity, with evidence of interstitial fibrosis in all patients, and areas of replacement fibrosis in over 60%. These results corresponded with those of Bernaba et al., who identified similar proportions of cardiac fibrosis within their patient cohort (Bernaba et al., 2010).

As Ewer et al. demonstrated, patients receiving anthracycline chemotherapy may display a normal cardiac ejection fraction, despite advanced histological changes, including myocardial fibrosis, present on cardiac biopsy (Ewer et al., 1984). The enhanced sensitivity of cardiac magnetic resonance imaging (CMR) has therefore provided clinicians with the capability to recognise these anthracycline-induced histological changes prior to the development of cardiac dysfunction, whilst avoiding the risks associated with cardiac biopsy. Therefore, screening for reductions in LV mass and changes in myocyte architecture throughout anthracycline administration may serve as a useful clinical biomarker in for the detection of early anthracycline-induced cardiotoxicity. To place into context findings of myocardial fibrosis, late-gadolinium enhancement (LGE) can be applied to CMR to detect focal myocardial scarring and fibrosis. This facilitates the differentiation between causes of cardiomyopathy (ischaemic vs non-ischaemic), provides assessment of myocardial viability, and can potentially

identify a range of chemotherapy-induced cardiotoxicities causing inflammatory or infiltrative disease. Following anthracycline exposure, progressive myocardial fibrosis derives from the cardiomyocyte damage and subsequent cardiac remodelling. Despite knowledge of the pathological processes and supporting results from animal models indicating increased LGE may predict subsequent reductions in LVEF, LGE has unfortunately not been shown to be the consistent imaging biomarker for the detection of anthracycline-induced cardiotoxicity in patients with cancer (Neilan et al., 2012, Thavendiranathan et al., 2013). Given that LGE is calculated based upon the focal cardiac fibrosis relative to normal myocardium, the degree of myocardial fibrosis may actually be underestimated in cases of extensive myocardial injury. Further research is required in this area to determine the full utility of LGE in patients receiving anthracycline chemotherapy and determine whether it has any prognostic role in early detection of anthracycline-induced cardiotoxicity. Further to the interstitial and replacement fibrosis observed within the myocardium, the histological analysis also identified sub-epicardial fibrosis within 3 patients. This localisation of fibrosis has not previously been detected on cardiac biopsy sampling or CMR in patients with anthracycline-induced cardiotoxicity, with this atypical pattern of fibrosis only rarely seen in cases of myocarditis and associated with a more insidious development of cardiac failure (Shimizu et al., 2000, Mahrholdt et al., 2006, Lota Amrit et al., 2021).

Overall, these advancements in CMR demonstrate its capability to detect the anthracycline-induced cardiotoxicity histological findings described within the patient cohort. Given the chronicity of the patients' diagnosis of anthracycline-induced cardiotoxicity and duration to cardiac transplantation, early features of anthracycline-induced cardiotoxicity such as myocardial inflammation and necrosis were not detected on histological sampling. Intermediate features anthracycline-induced cardiotoxicity confirmed with the presence of myocytolysis were detected in almost half of patients. Whilst late anthracycline-induced cardiotoxicity features were observed in all patients with varying grades of interstitial fibrosis, whilst almost two thirds of patients displayed replacement fibrosis. This study therefore provides valuable insight into the spectrum anthracycline-induced cardiotoxicity histopathology and describes the pathway of myocardial changes in response to anthracycline chemotherapy. Future histopathology and CMR studies should also consider monitoring for sub-epicardial changes, which have first been detected within this study of patients with confirmed anthracycline-induced cardiotoxicity, holding the potential to strengthen our understanding of anthracycline-induced cardiotoxicity and cardio-protection in these patients.

3.9.1 Conclusions

In summary, two pre-clinical models, AC10 cardiomyocytes and primary neonatal rodent ventricular cardiomyocytes, have been characterised and qualified, establishing the time and concentration-dependent cardiomyocyte responses to doxorubicin. These results demonstrate the strong influence of anthracycline dose and cumulative exposure upon cardiomyocyte viability, as the acute cardiomyocyte responses to doxorubicin were evaluated with xCELLigence technologies. This emphasises the importance of considering initial blood plasma concentrations and drug pharmacokinetics in patients receiving anthracycline chemotherapy, especially as reduced rates of drug distribution, protein binding and clearance, are known to significantly increase systemic free drug levels and subsequently myocardial exposure. The histological analyses within cardio-oncology patients indicates the pressing need for pre-clinical studies evaluating the doxorubicin-sensitive responses of individual cell types within the myocardium, specifically fibroblasts and cardiac progenitor cells. Key findings from these studies will promote the discovery of novel cardiotoxicity biomarkers and cardioprotective strategies. As paediatric and adult hearts exist in distinct phases of myocardial development, the composition of cells represented within the myocardium differs. These characteristics strongly determine the cardiomyocyte responses to anthracycline exposure, and signals the clinical progression of cardiotoxicity.

The next stage is to consider how these pathological responses influence the myocardial compensatory mechanisms. Neurohumoral pathways are activated in response to cardiomyocyte injury, with the renin-angiotensin-aldosterone system identified as a prime therapeutic target for anthracycline-induced cardiotoxicity prevention. The enhancement of pharmacological strategies to limit clinical drug-induced toxicity, whilst retaining anti-cancer therapeutic efficacy, and identifying ‘at risk’ patients could prevent irreversible anthracycline-induced cardiotoxicity.

Chapter 4. Evaluation of the involvement of the angiotensin signalling pathway in anthracycline-induced cardiotoxicity

4.1 Introduction

As long-term cancer survivorship continues to improve, the need to recognise, manage and mitigate acute and chronic anthracycline-induced cardiotoxicity becomes increasingly important, especially as early detection can prevent cardiovascular morbidity and mortality for these patients. To maximise long-term survival and reduce cardiovascular complications, a comprehensive understanding of the pathological mechanisms culminating in anthracycline-induced cardiomyocyte injury and structural toxicity are required. In addition to enhancing detection and guiding effective preventative strategies, special attention should be focussed on understanding subclinical toxicity, as early pharmacological intervention has been shown to preserve cardiac function and promote recovery (Cardinale et al., 2010).

Cumulative anthracycline dose is regarded as a substantial risk factor for cardiovascular toxicity, with the incidence of dose-dependent congestive cardiac failure recorded as 4.7%, 26%, and 48% for anthracycline doses of 400mg/m^2, 550mg/m^2, and 700mg/m^2, respectively (Swain et al., 2003). Further to cumulative dose, the analysis of endomyocardial biopsy samples taken after anthracycline therapy demonstrated that peak plasma levels were also associated with clinically significant cardiotoxicity (Legha et al., 1982). In response to these findings, novel anthracycline formations and optimal dosing schedules have been developed and are now incorporated within modern-day chemotherapy protocols, limiting peak plasma levels whilst maintaining maximal therapeutic efficacy. Meta-analysis data also supports the inclusion of age, female gender, cardiovascular co-morbidities, hypertension and chest radiotherapy within predictive and prognostic cardiotoxicity algorithms (Lotrionte et al., 2013, Zhang et al., 2022). However, analyses have shown that despite optimal control of these known risk factors, patients still develop cardiotoxicity. This infers both the existence of undetected risk factors, and an even stronger potential for genetic variants influencing cardiovascular responses to anthracycline exposure. Moreover, long-term retrospective cohort analyses have shown crucially that even patients considered to have received low-dose anthracycline therapy (<250mg/m^2) develop the hallmarks of cardiotoxicity, many years after anthracycline treatment, especially as future cardiovascular stressors can reveal the previously undetected subclinical myocardial damage (Kremer et al., 2001, Mulrooney et al., 2009).

Despite a firm understanding of the anti-cancer pharmacological properties of anthracyclines, which translates into excellent therapeutic efficacy, our understanding of the mechanisms behind cardiotoxicity remains limited. Multiple hypotheses involving reactive oxygen species synthesis, topoisomerase II-linked DNA damage, and the role of iron complex formation have been postulated to explain the cardiotoxic mechanism or mechanisms of anthracyclines, linked to an initial loss of cardiomyocytes from the myocardial mass. This acute cardiomyocyte and myocardial response following anthracycline exposure is determined by the degree of cardiomyocyte damage. When only minor myocyte injury is sustained, typically within the early cycles or after low doses of chemotherapy, histological analysis may reveal cytoplasmic vacuolisation with accompanying oedema, inflammatory cell infiltration and cardiomyocyte degeneration (Cove-Smith et al., 2014). In response to this cardiomyocyte damage, cardiac troponins located within the contractile apparatus of cardiac muscle are released and enter the systemic circulation, providing a biomarker-driven measure of this cardiac damage (White, 2011, Apple and Collinson, 2012). Studies now indicate that elevations of cardiac damage biomarkers (i.e. cardiac troponins) in anthracycline-induced cardiotoxicity conveys a higher risk of developing symptomatic cardiotoxicity with a reduced life expectancy (Cardinale et al., 2004, Jones et al., 2017, Michel et al., 2018).

Despite these initial toxicity-driven losses in cardiac cells, it is the fate of the remaining cardiac cells and the impact of sub-therapeutic exposures of anthracyclines upon the heart, which are central to the development of delayed late-onset cardiotoxicity. The loss of cardiomyocytes from the heart will inevitably lead to a reduction in peak myocardial force and cardiac output, with the remaining cardiomyocytes placed under increased mechanical stress as they attempt to compensate for the cardiac damage incurred (Guyton and Hall, 2011). Crucially, most patients remain asymptomatic during these early phases. However, over time, changes in physiological parameters and cardiac imaging begin to reveal a decline in the cardiac pumping capacity, termed ventricular dysfunction. Ultimately, these patients with ventricular dysfunction are likely to become symptomatic, associated with high rates of morbidity and mortality (Mann and Bristow, 2005). The driving forces behind this progressive ventricular dysfunction and cardiac failure relate to compensatory sympathetic stimulation, neurohormonal activation and subsequent cardiomyocyte hypertrophy, in an attempt by the heart to compensate for haemodynamic pressure and volume overload. One molecular pathway which is integral to these processes is the angiotensin signalling pathway (described in section 1.6.2). Dysregulation of this pathway has been implicated in anthracycline-induced

cardiotoxicity, evidenced by the ability of pharmacological inhibition of the pathway to reduce the degree of heart failure associated with anthracyclines (Cardinale et al., 2006). The precise mechanism of the angiotensin signalling pathway in relation to cardiomyocyte responses to anthracyclines are yet to be determined. Furthermore, research is required to establish the relationship between the physiological and pathological processes of angiotensin-driven haemodynamic control in the presence of anthracyclines, and the potential for therapeutic intervention.

4.1.1 Angiotensin regulation and dysregulation

The principal mechanism of the angiotensin signalling pathway is to support regulation of blood pressure and maintain end-organ perfusion, through the renin-angiotensin-aldosterone system (RAAS). This pathway is stimulated in response to low arterial pressure and is initiated by renin secretion from within the juxtaglomerular cells of the kidneys. Renin acts on the globulin angiotensinogen, releasing angiotensin I into the circulation. Angiotensin I is only a mild vasoconstrictor, therefore angiotensin converting enzyme (ACE) cleaves this peptide molecule into angiotensin II. Angiotensin II is a powerful hormonal vasoconstrictor, directly increasing arterial pressure through widespread small arteriole constriction. Acute rises in circulating angiotensin II therefore promotes systemic vasoconstriction, producing transient decreases in tissue blood flow and increased total peripheral resistance. In addition to its vasoconstrictive properties, angiotensin II reduces renal salt and water excretion. Through direct renal arteriolar vasoconstriction and promotion of aldosterone secretion from the adrenal glands, the RAAS expands extracellular volume to further increase arterial pressure. By optimising haemodynamic control, these physiological responses maintain organ perfusion even in low circulating volume states such as hypotension or hypovolaemia (Atlas, 2007, Guyton and Hall, 2011).

The physiological effects of angiotensin II help maintain circulatory homeostasis and prevent end-organ damage. Activation of G-protein coupled receptors, termed angiotensin II receptor type 1 (AT1R), produces vasoconstriction, cardiomyocyte growth, sympathetic stimulation, and increases myocardial contractility. These effects initially promote cardiovascular growth and remodelling, however, overstimulation of the RAAS and prolonged exposure to angiotensin II can eventually lead to the development of hypertension and associated cardiovascular morbidity. Dysregulation of the RAAS mechanisms and excessive angiotensin II production are strongly associated with clinical hypertensive disorders. Chronic RAAS

stimulation encourages hyperplasia and hypertrophy of vascular smooth muscle cells, with the resulting increase in vascular tone elevating arterial blood pressure. High vascular tone has secondary effects upon the heart, with greater cardiac contractile forces required to overcome the increased peripheral vascular resistance. Consequently, cardiomyocyte hypertrophy soon develops, and if unregulated can lead to the development of cardiac failure due to excessive intra-cardiac pressures.

4.1.2 Angiotensin responses to cardiovascular pathology

Hypertension is a recognised major risk factor for the development of cardiovascular disease including heart failure, ischaemic heart disease, stroke, atrial fibrillation, valvular heart disease and chronic kidney disease (Kannel, 2000, Fuchs and Whelton, 2020). The principal function of the RAAS is blood pressure regulation, driven by renal angiotensin II and adrenal aldosterone, together with local angiotensin II formation. Hypertension is strongly associated with RAAS overactivation, therefore pharmacological therapies targeting the RAAS pathway are widely used to treat hypertension and minimise cardiovascular risk (López-Sendón et al., 2004, Atlas, 2007, Williams et al., 2018).

Chronic RAAS activation increases peripheral vascular resistance by systemic vasoconstriction and vascular remodelling. These changes increase haemodynamic pressure load combined with physiological wall stress, promoting left ventricular cardiomyocyte hypertrophy. In addition to cardiomyocyte adaptation, tissue remodelling within the extracellular matrix accelerates cardiac hypertrophy, combining increased fibroblast turnover with excess collagen formation. Whilst these adaptions maintain stroke volume in the short-term, interstitial fibrosis propagates, with the resulting ventricular wall stress causing ventricular dilatation and thinning (Lorell and Carabello, 2000, Crowley et al., 2006). These adverse remodelling changes are further exacerbated by inflammation and increases in reactive oxygen species (ROS), formed in conditions of excess angiotensin II such as hypertension and heart failure (Mehta and Griendling, 2007, Benigni et al., 2010). The resulting reduction in cardiomyocyte structural support and continued haemodynamic overload renders patients susceptible to cardiac decompensation. Furthermore, supporting data from the Framingham study, a long-term observational cohort study into cardiovascular disease, has suggested left ventricular hypertrophy (LVH) has significant prognostic implications, increasing the risk of cardiovascular morbidity and mortality (Levy et al., 1990, Haider et al., 1998, Benjamin and Levy, 1999).

Marked elevations in angiotensin II levels are demonstrated after myocardial infarction, in response to pressure and volume overload caused by the acute reduction in cardiac function. Although this cardiovascular research has been predominantly conducted in animal experimental models, RAAS activation associated with increases in AT1R binding density and acute elevations in angiotensin II post-myocardial infarction have been observed. Studies evaluating ^{125}I-labelled angiotensin kinetics have established the cardiac localisation of angiotensin II, with angiotensin II detectable on myocytes and interstitial cells (Serneri et al., 1996, Leenen et al., 1999, van Kats et al., 2000, Serneri et al., 2001). In the case of myocardial infarction, angiotensin II binding is detectable within the infarcted regions, which quickly become established with fibroblast proliferation and collagen deposition (Sun and Weber, 1994, Lefroy et al., 1996, Yang et al., 1997). Therefore, increases in angiotensin II concentration are not only a direct response to cardiac pressure and volume overload, but angiotensin II localisation is associated with increases in inflammation, oxidative stress and fibrosis following infarction. Oxidative stress is clinically significant because the formation of reactive oxygen species (ROS) induces mitochondrial dysfunction, cardiac hypertrophy and fibrosis. Allowing unregulated progression of these mechanisms leads to adverse cardiac remodelling and permanent cardiac impairment (Crowley et al., 2006, Xu et al., 2010, Tsutsui et al., 2011).

4.1.3 Associations between angiotensin converting enzyme (ACE) gene polymorphisms and cardiomyopathies

The angiotensin converting enzyme (ACE) gene (17q23.3 *locus*) codes for the enzyme, with RAAS activation strongly influenced by genetic ACE expression (Rigat et al., 1990). The presence of a 287bp insertion/deletion polymorphism in intron 16 of the ACE gene has been strongly correlated with overall gene expression levels of ACE, and subsequent serum ACE levels (Rigat et al., 1990). Of the potential genotypes, the homozygous deletion genotype (D/D) has been shown to produce the highest concentrations of serum and intracardiac ACE, with mean serum ACE measuring up to twice the concentration of the I/I genotype, with the I/D genotype recording intermediate values (Rigat et al., 1990, Tiret et al., 1992, Danser et al., 1995, Biller et al., 2006). Furthermore, this specific ACE gene polymorphism has been linked to several cardiovascular conditions and ventricular remodelling associated with RAAS activation (Cambien et al., 1992, Schunkert et al., 1994, Zintzaras et al., 2008, Yuan et al., 2017, Liu et al., 2021).

Several meta-analyses have explored this relationship between cardiovascular pathology and the ACE gene polymorphism. Liu et al. (2021) demonstrated that deletion of the ACE allele is associated with a higher susceptibility of developing essential hypertension (Liu et al., 2021). Expression of this D/D genotype was identified as an independent cardiovascular risk factor, adversely influencing blood pressure regulation and cardiovascular remodelling, with males and those of Asian origin most affected (Liu et al., 2021). These findings of a relationship between the ACE gene and cardiovascular disease are supported by Zintzaras et al. (2008) who showed an association between the I/D polymorphism variant and a 25% increase in coronary artery disease (Zintzaras et al., 2008), whilst a meta-analysis conducted by Chen et al. (2013) indicated that the deletion allele was associated with an increased risk of myocardial infarction (Chen et al., 2013). Furthermore, Yuan et al. (2017) emphasised the impact of angiotensin II formation upon cellular proliferation, cellular hypertrophy, and ventricular remodelling, with their meta-analysis confirming the association between the ACE gene and myocardial hypertrophy (Yuan et al., 2017). The expression of the I/D polymorphism was a significant genetic risk factor for the development of hypertrophic cardiomyopathy, suggesting that ACE inhibitor or ARB therapy holds the potential to modify the clinical phenotype for hypertrophic cardiomyopathy (Yuan et al., 2017). However, one important caveat is that clinical studies have shown significant clinical interactions exist between ACE genotype polymorphisms and ACE inhibitors responses in patients with both hypertension and heart failure. These studies therefore underline how patient cardiovascular responses to angiotensin inhibition are not uniform, and are susceptible to modification by RAAS genetic variants (O'Toole et al., 1998, Scharplatz et al., 2005, Heidari et al., 2017).

4.1.4 Anthracycline induced cellular responses and angiotensin signalling

Clinical studies have clearly indicated a relationship between perturbation of the angiotensin signalling pathway and development of anthracycline-induced cardiotoxicity (Cardinale et al., 2006, Cadeddu et al., 2010, Janbabai et al., 2017). However, as previously stated, the mechanisms underlying this are unclear.

Several studies have supported the induction of cardiomyocyte hypertrophy and upregulation in hypertrophy markers following exposure to anthracyclines, an effect that continues to occur at sub-clinical and negligibly cytotoxic concentrations. This observed response is likely to be a rection to perturbations of molecular signalling and survival pathways for the myocardium (Rockley and Gill, 2017, Yoon et al., 2020, Jefferies et al., 2021, Leerink et al., 2021). In support

of a linkage between the physiological cardiovascular response to angiotensin and the pathological responses observed with anthracyclines in cardiac cells, similarities have been observed in relation to a pro-hypertrophic response, with increased mitochondrial and tubular size, sarcoplasmic reticular dilatation, cardiomyocyte hypertrophy, and subsequently myocardial fibrosis (Unverferth et al., 1981, Mortensen et al., 1986, Vásquez-Trincado et al., 2016). One strong hypothesis for this relationship is that anthracyclines stimulate and/or induce the angiotensin response in cardiomyocytes, thereby promoting cellular hypertrophy. Both anthracyclines and angiotensin II can induce oxidative stress and stimulate reactive oxygen species formation within mitochondria (Sachse and Wolf, 2007, Simůnek et al., 2009, Dikalov and Nazarewicz, 2013). Accumulation of reactive oxygen species culminates in myocardial injury and increased haemodynamic stress, leading to the formation of cardiac hypertrophy (Maulik and Kumar, 2012, Burchfield et al., 2013). This relationship is supported by results of *in vivo* animal studies, which have demonstrated both the induction of cardiomyocyte hypertrophy and interstitial fibrosis caused by anthracycline exposure (Okumura et al., 2002, Adıyaman et al., 2022). These changes were significantly improved by the administration of RAAS inhibitors (Okumura et al., 2002, Iqbal et al., 2008, Arozal et al., 2010, Taskin et al., 2016), whilst doxorubicin-induced cardiomyocyte injury was prevented entirely in angiotensin II type-1 receptor knockout mice (Toko et al., 2002), signifying direct influence of the angiotensin II signalling pathway upon anthracycline-induced cardiotoxicity.

An alternative hypothesis for the induction of cellular hypertrophy by anthracyclines is that it results from paracrine signalling from fibroblasts, endothelial cells, and cardiac progenitor cells (Piegari et al., 2016, Cappetta et al., 2018). The mechanisms for these signalling cascades may not only originate from angiotensin II release, but could instead be stimulated by oxidative stress, DNA damage, cell death and cellular senescence (Sadoshima and Izumo, 1993, Gray et al., 1998, De Angelis et al., 2016). Following exposure to anthracyclines, adverse cellular changes provoke mechanical cardiac stress and create haemodynamic overload. The release of endothelin-1 for example, a powerful vasoconstrictor, promotes cardiomyocyte hypertrophy through induction of hypertrophic gene expression, leading to myocardial remodelling (Archer et al., 2017). Peptide growth factors (e.g. tumour growth factor-β1) and the local production of inflammatory cytokines (e.g. interleukin-1β) released within the cardiomyocytes and surrounding tissues, similarly enhance cardiomyocyte hypertrophy, the latter also inducing interstitial fibrosis formation. Whilst adaptive in the short-term, chronic exposure to these remodelling mechanisms eventually renders them ‘maladaptive’, as

fundamental changes to myocardial composition reduces contractile efficiency, increasing biomechanical stress and culminates in cardiac failure (Colucci, 1997). Irrespective of the responsible pathway, a role for angiotensin signalling specifically upon cardiomyocytes has been strongly implicated, but as yet unproven.

The relationship between the ACE polymorphic phenotype and development of cardiomyopathies analogous to anthracycline-induced cardiotoxicity also creates a potential linkage between these pathways and cardiotoxicity. Activation of the RAAS pathway and subsequent physiological responses is strongly influenced by genetic ACE expression (Rigat et al., 1990, Tiret et al., 1992, Danser et al., 1995, Biller et al., 2006). As such, since angiotensin II formation is dependent upon ACE activity levels, gene expression driven increases in ACE activity are associated with the increased levels of angiotensin II. Higher levels of angiotensin II would consequently drive a greater physiological response (Benigni et al., 2010, Zhang et al., 2017). In the context of anthracycline-induced cardiotoxicity, such an association may further perpetuate the response by pushing cardiomyocytes over a tolerance threshold toward hypertrophy, compensatory processes, and subsequent cardiac dysfunction. Potentially, patients exhibiting the ACE insertion genotype, associated with lower ACE activity, in the presence of anthracyclines would not generate sufficient angiotensin II to trigger the negative effects. In contrast, patients with higher baseline angiotensin II levels in the presence of anthracycline-stimulated elevations in angiotensin II or AT1R, may increase cellular responses and downstream effects. In both putative scenarios, the mechanism responsible and involvement of angiotensin signalling is obviously yet to be determined.

4.1.5 Chapter aims and objectives

The aim of this chapter is to ascertain the relationship between the angiotensin signalling pathway and the detrimental cardiac effects of anthracyclines to identify clinical strategies to mitigate this toxicity. This will be achieved through the following objectives:

1. Evaluate the effects of clinically relevant doxorubicin concentrations upon angiotensin II type-1 receptor (AT1R) gene and protein expression within the AC10 human ventricular cardiomyocyte cell line

2. Quantify the presence and influence of doxorubicin upon angiotensin II levels in AC10 human ventricular cardiomyocyte cell lines *in vitro*

3. Analyse the plasma angiotensin II concentrations and ACE genotypes of patients diagnosed with breast cancer receiving anthracycline chemotherapy, identifying the relationship between plasma angiotensin II concentrations, ACE genotyping, and the cardiotoxicity phenotype

4. Determine whether plasma angiotensin II concentrations and ACE genotype are predictive of the development of anthracycline-induced cardiotoxicity, to ascertain the potential benefit from ACE inhibitor therapy during anthracycline treatment

4.2 Results

4.3 Analysis of angiotensin II type-1 receptor (AT1R) gene expression following exposure to sub-therapeutic concentrations of doxorubicin

4.3.1 Establishing the qRT-PCR standard curve analysis and reference gene optimisation prior to analysing AT1R gene expression within AC10 cardiomyocytes treated with doxorubicin

One potential issue identified in determination of AT1R gene expression was the appropriateness of GAPDH as a 'housekeeping' standardisation control (Backlund et al., 2009). To address this, the additional reference genes selected for comparison were ribosomal protein L13a (RPL13A), hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1), and TATA-Box Binding Protein (TBP). Using a serial cDNA dilution of the 'control' sample, sample purities and primer efficiencies were evaluated, and a standard curve generated for each of the reference genes derived from the qRT-PCR cycle quantification (Cq) values as calculated by the QuantStudio 7 software.

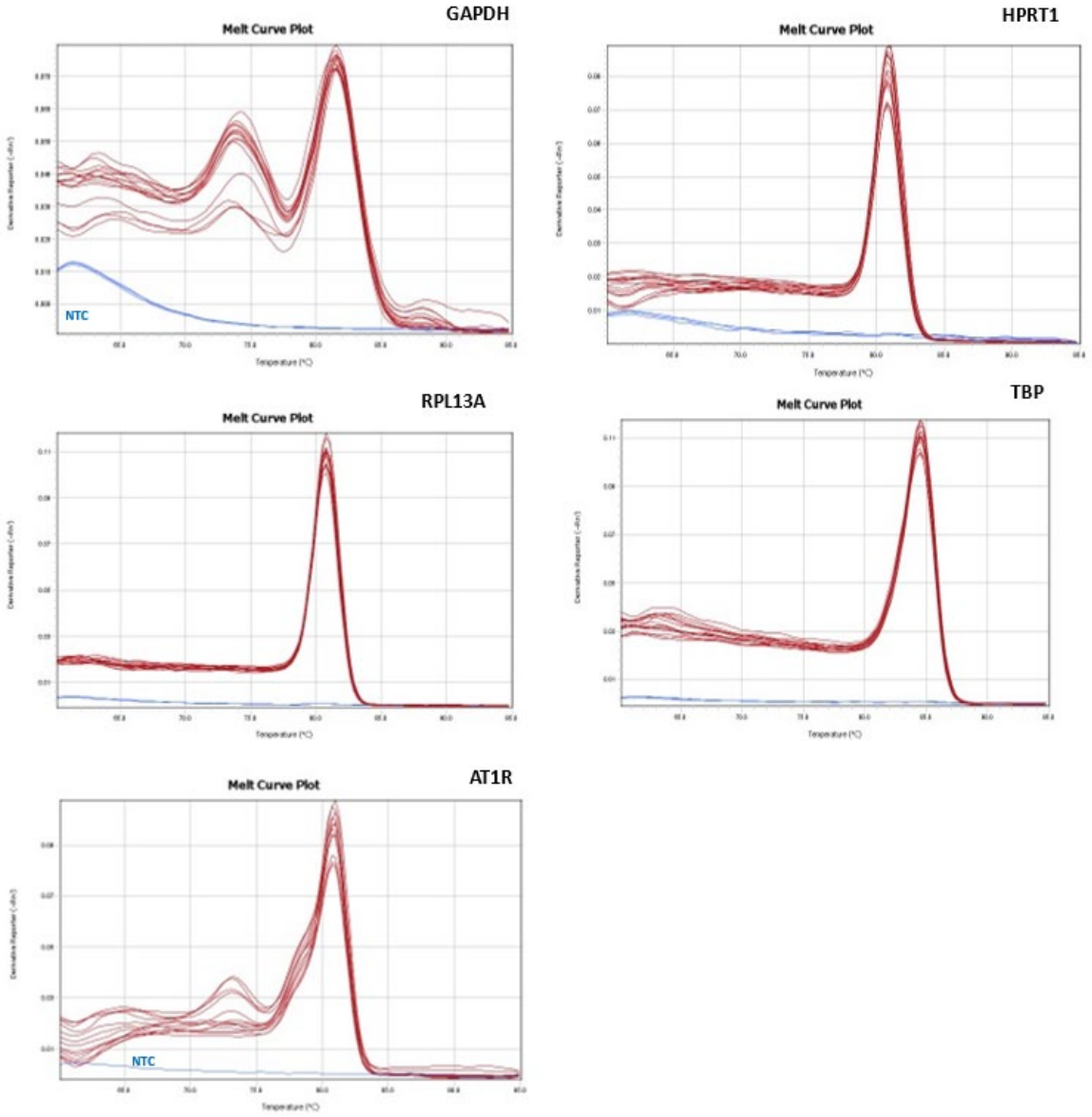


Figure 4.1 **Melt curve plot analysis of reference genes after 40 cycles to evaluate the specificity of each amplified reaction.** Dissociation analysis was performed by heating the amplicon from 60 to 95°C. NTC = no-template control (absence of cDNA).

The results of the melt curve analysis (**Figure 4.1**) demonstrate singularly peaked dissociation curves for HPRT1, TBP and RPL13A reference genes, indicating single, specific products with no primer dimers produced in either the DNA samples or no-template controls. The GAPDH dissociation curve, however, reveals the presence of an initial peak at 74°C, with a larger peak following at 82°C suggesting the presence of 2 amplicons. The larger peak represents the expected amplicon, whilst the initial peak is likely to represent either primer dimer formation or the relative stabilities of the GAPDH reaction products. Analysis by gel electrophoresis

confirmed the presence of primer dimers, making GAPDH unsuitable for the subsequent qRT-PCR experiments as a standardised control.

Serial cDNA dilutions were used to produce standard curves for each of the reference genes (**Figure 4.2**), with correlation coefficients (r^2) and amplification efficiencies calculated (**Table 4.1**). Of the four reference genes evaluated, only TBP did not satisfy the optimisation criteria of $r^2 > 0.98$ and amplification efficiency 90-110%, with efficiency calculated as 112%.

Table 4.1 Reference gene amplification efficiencies

Gene	Slope	Y-intercept	r^2	Efficiency (100%)	Standard Error	Melting Point (Tm1)
AT1R	-3.001	27.37	0.976	115.41	0.129	80.84
GAPDH	-3.370	25.081	0.996	98.036	0.062	81.506
HPRT1	-3.407	24.390	0.996	96.564	0.059	80.811
TBP	-3.055	25.321	0.992	112.492	0.075	84.524
RPL13A	-3.136	16.160	0.995	108.395	0.06	80.767

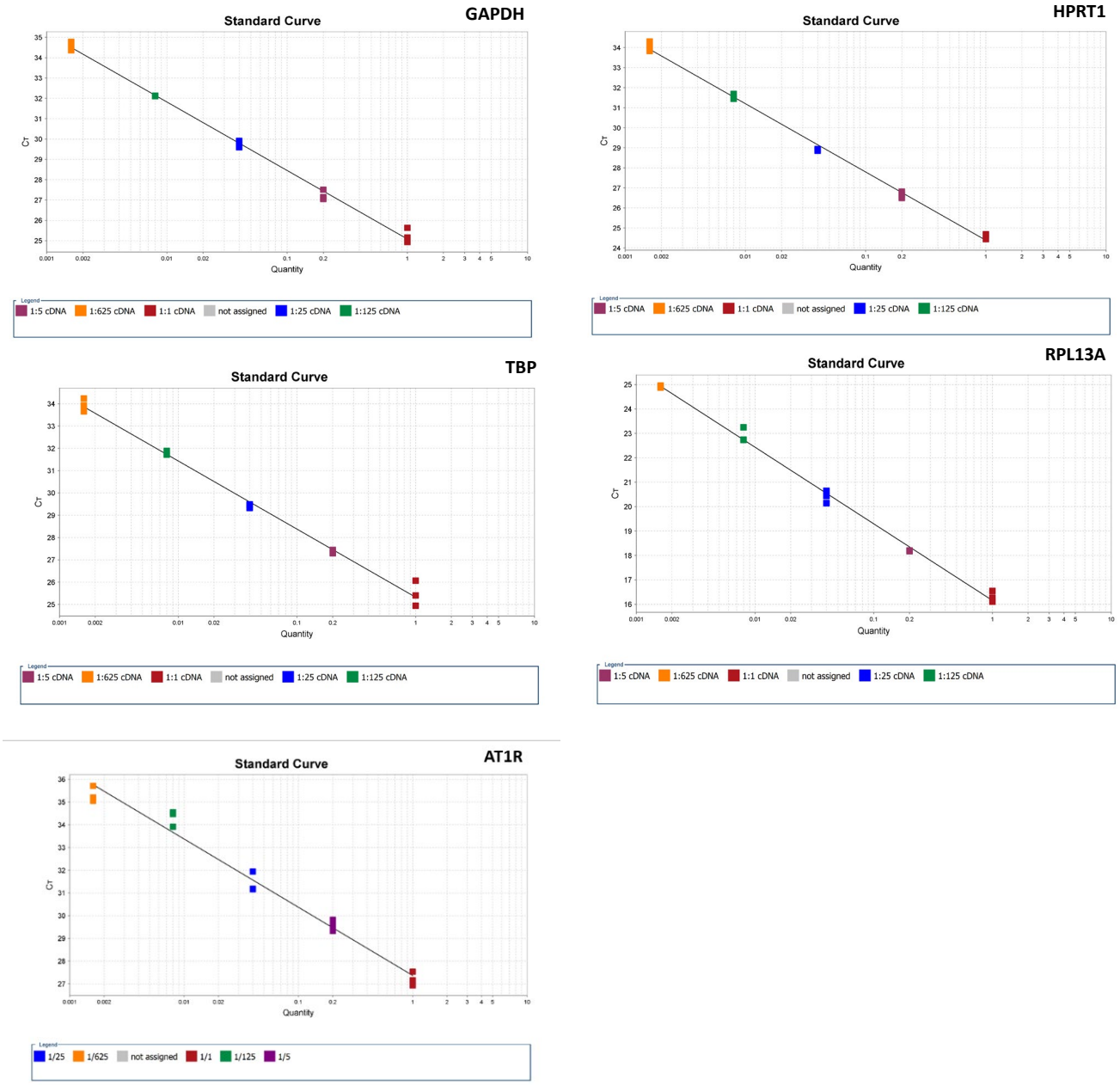


Figure 4.2 **Standard curves for each reference gene produced by serial cDNA dilutions and qRT-PCR analysis.**

To strengthen this stability assessment, the individual Cq values were then extracted from the standard curve analyses and used to directly compare the reference genes against one another (**Figure 4.3**), enabling the genes to be ranked in order of stability and providing a more comprehensive assessment. Using the RefFinder statistical algorithms (http://www.leonxie.com/referencegene.php), four qRT-PCR normalisation algorithms (BestKeeper, Genorm, Delta CT method and normFinder) were used to compare the raw data from the standard curve experiments and subsequently rank the reference genes in order of stability (appendix B) (Vandesompele et al., 2002, Andersen et al., 2004, Pfaffl et al., 2004,

Silver et al., 2006). A comprehensive ranking of the reference genes was established, with RefFinder combining the evaluations of the four normalisation algorithms into a single table (**Table 4.2**). This demonstrated that overall, RPL13A was the most stable reference gene, followed by HPRT1 and GAPDH, whilst TBP was the least stable.

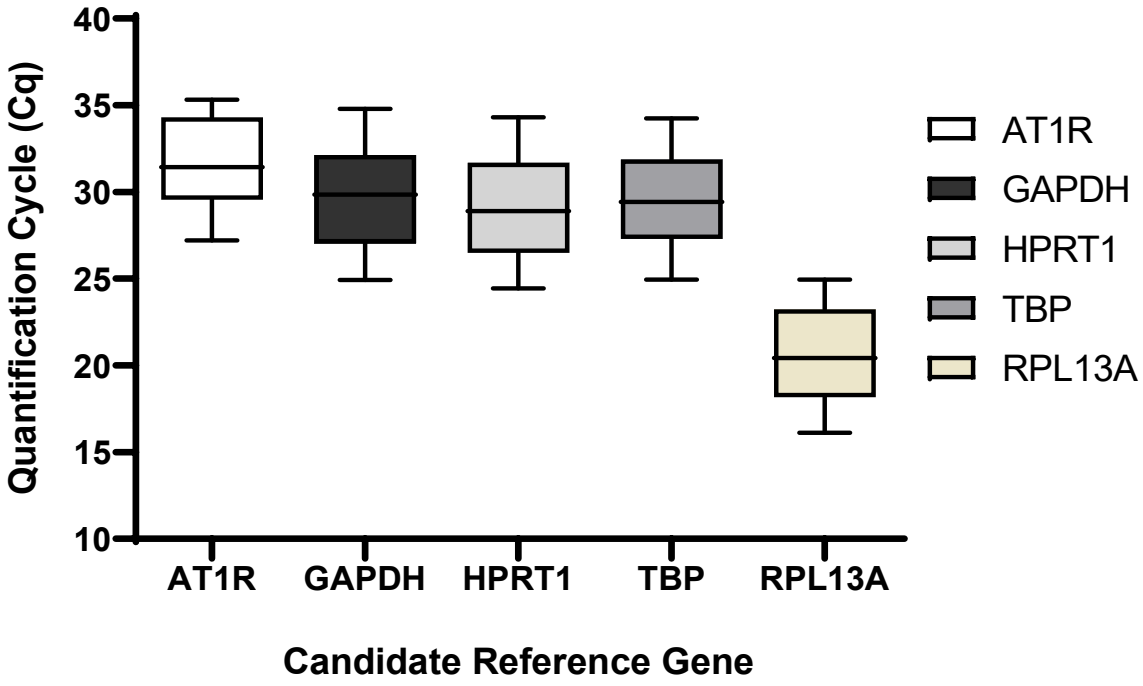


Figure 4.3 **Expression levels of candidate reference genes compared to AT1R, calculated using qRT-PCR QuantStudio.** The boxes demonstrate quantification cycle (Cq) values, with the median represented by the central horizontal line. The upper and lower quartiles are represented by the box, whilst the whiskers denote the minimum and maximum values.

Table 4.2 **Comprehensive ranking of candidate reference genes**

Ranking Order (Better--Good--Average)				
Method	1	2	3	4
Delta CT	RPL13A	HPRT1	GAPDH	TBP
BestKeeper	TBP	RPL13A	GAPDH	HPRT1
Normfinder	RPL13A	HPRT1	GAPDH	TBP
Genorm	GAPDH | HPRT1	-	RPL13A	TBP
Recommended comprehensive ranking	**RPL13A**	HPRT1	GAPDH	TBP

4.3.2 Reference gene analysis and selection following AC10 cardiomyocyte exposure to sub-therapeutic concentrations of doxorubicin

The influence of doxorubicin exposure on AT1R expression was calculated by normalising qRT-PCR Cq values against a suitable endogenous control, to represent AT1R expression as a fold change relative to control. The gene expression levels of AT1R were normalised to the mean of the reference genes, the results are represented in **Figure 4.4** and **Table 4.3**.

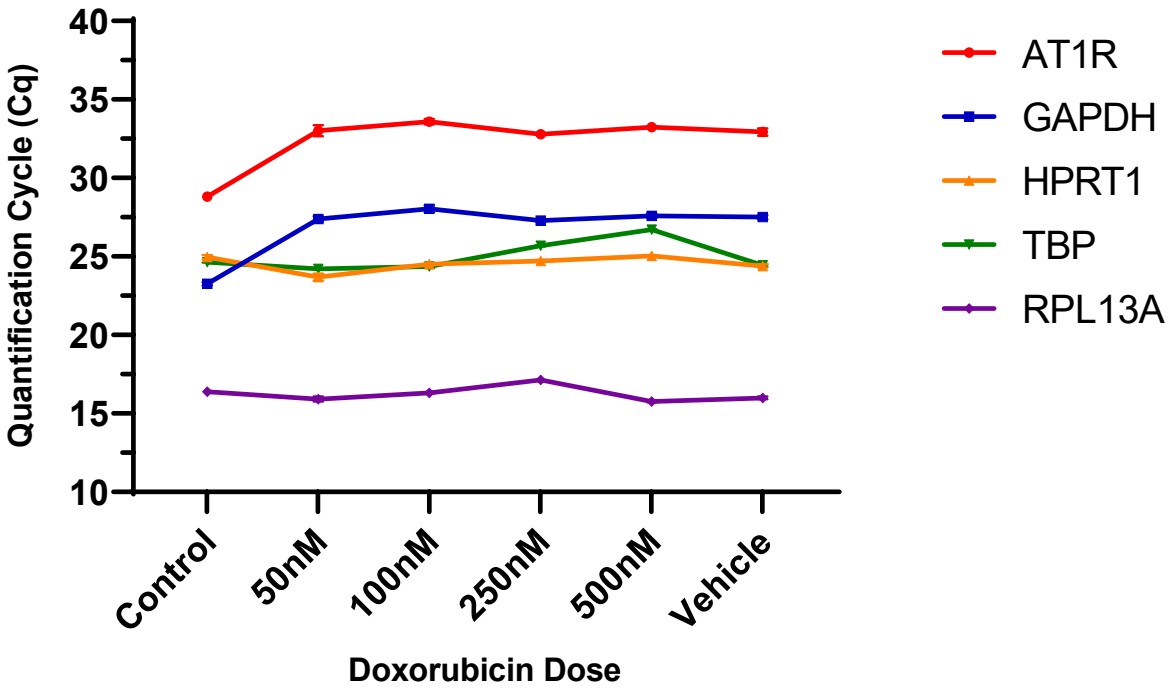


Figure 4.4 **Expression of reference genes following AC10 cell exposure to subclinical doses of doxorubicin for 24 hours for evaluation of gene stability**

Table 4.3 **Comprehensive ranking of candidate reference genes following 24 hours of doxorubicin exposure**

Ranking Order (Better--Good--Average)				
Method	1	2	3	4
Delta CT	HPRT1	RPL13A	TBP	GAPDH
BestKeeper	HPRT1	RPL13A	TBP	GAPDH
Normfinder	HPRT1	RPL13A	TBP	GAPDH
Genorm	HPRT1 | RPL13A		TBP	GAPDH
Recommended comprehensive ranking	HPRT1	RPL13A	TBP	GAPDH

Individual results were analysed using RefFinder, with comprehensive gene stability identifying HPRT1 as the most stable gene in samples treated with doxorubicin, followed by RPL13A, with TBP and GAPDH being the least stable. When cDNA samples were pre-treated with sequential doxorubicin doses prior to harvesting and analysis, HPRT1 was established to be the most stable reference gene, with RPL13A second with comparable stability values. TBP was revealed to be the least stable reference gene within the initial control cDNA samples, and demonstrated mid-range stability in the doxorubicin samples.

Overall, RPL13A was selected as the control reference gene for future qRT-PCR experiments, demonstrating the best stability for the control samples, and high stability within the doxorubicin-treated samples (**Figure 4.4** and **Table 4.3**).

4.4 Effects of sub-therapeutic concentrations of doxorubicin upon cellular expression of the AT1R gene

To establish the influences of doxorubicin dose and exposure time upon AT1R gene expression, AC10-CM in plateau phase of growth were exposed *in vitro* to a range of clinically relevant doxorubicin doses (50nM, 100nM, 250nM, 500nM) and durations (4-96 hrs) and AT1R expression determined by qRT-PCR **Figure 4.5** and **Figure 4.6**.

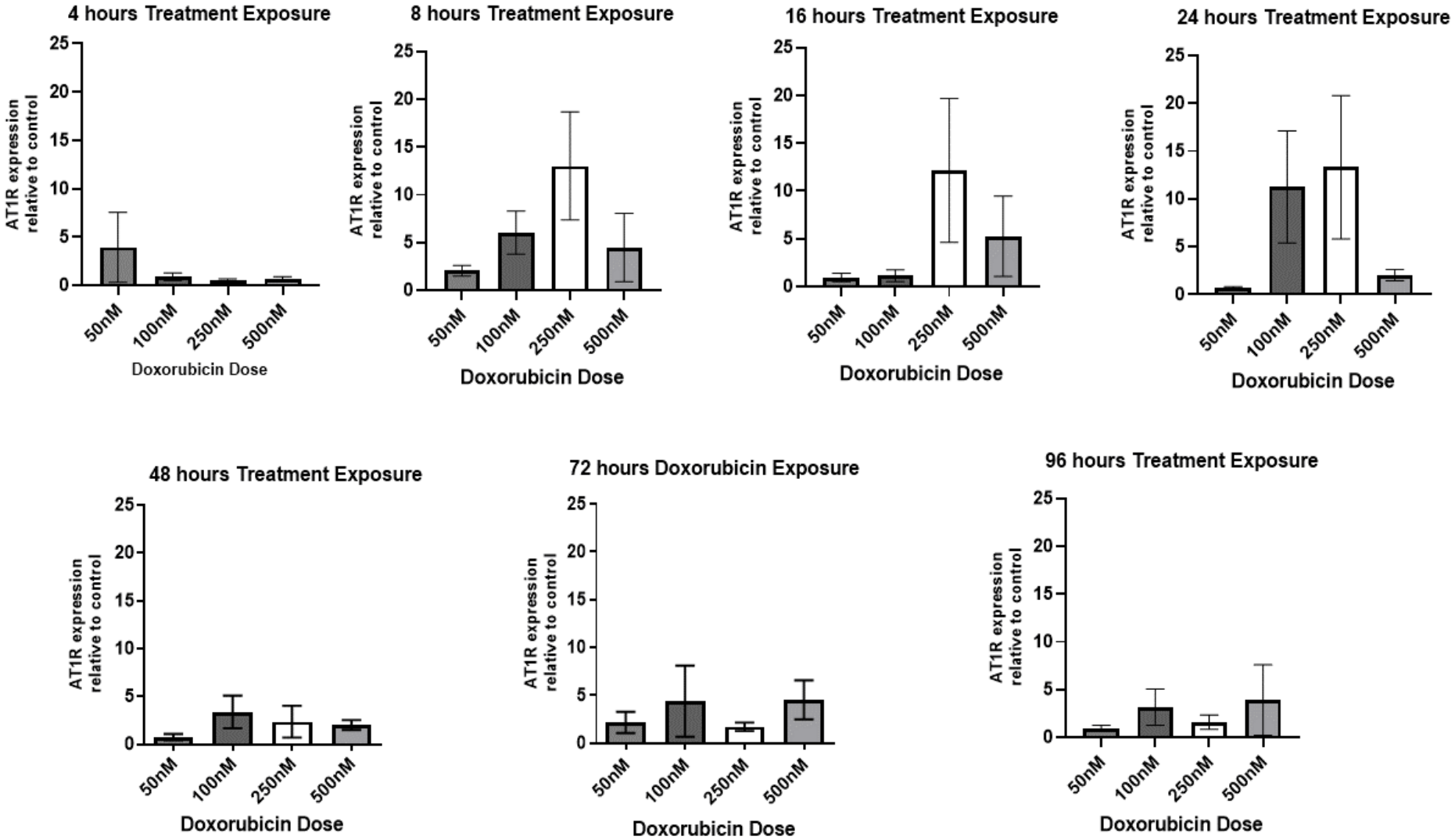


Figure 4.5 Effect of doxorubicin exposure upon gene expression of AT1R in AC10 cells in vitro. Exposure to clinically relevant doxorubicin concentrations (< Cmax) induces time and concentration dependent increases in AT1R gene expressions, determined by RT-qRT-PCR. Values are expressed relative to untreated controls (±S.E.) having normalised to RPL13A controls ($2^{\Delta\Delta}$Ct method). Results are representative of at least three experimental repeats.

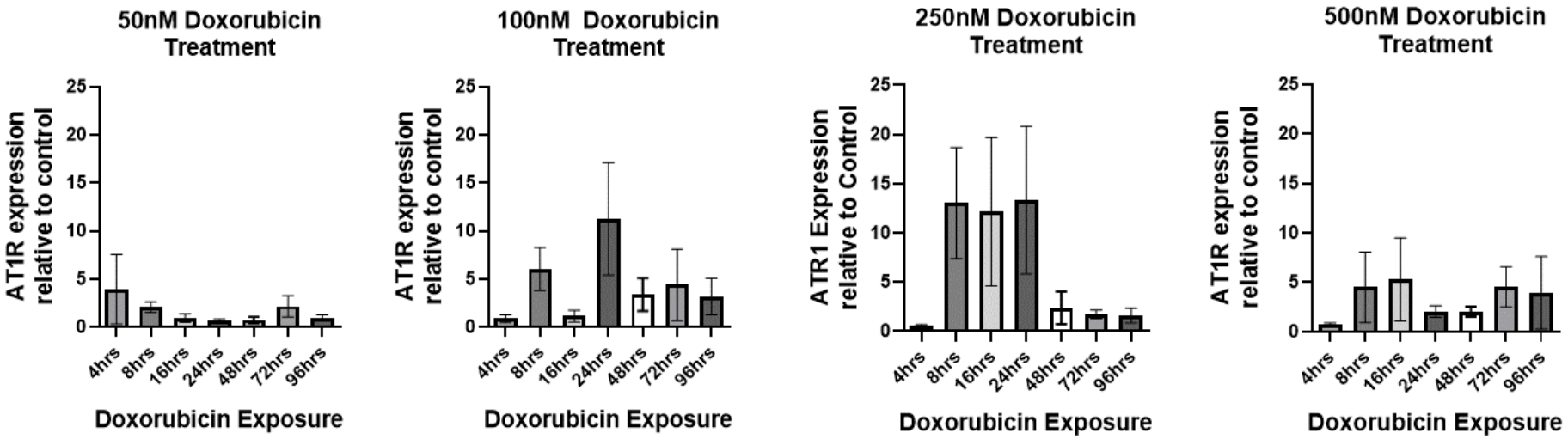


Figure 4.6 **AT1R gene expression in response to clinically relevant doses of doxorubicin in AC10 cell in vitro.** Values are expressed relative to untreated controls (±S.E.) having normalised to RPL13A controls ($2^{\Delta\Delta}$Ct method). Results are representative of at least three experimental repeats.

A positive correlation was observed between clinically relevant concentrations of doxorubicin and induction of gene expression of AT1R in AC10-CM cells, both concentration and exposure-time dependent (***Figure 4.5*** and ***Figure 4.6***). The highest levels of AT1R gene expression were observed from 8-24 hours of doxorubicin exposure, with doxorubicin doses of 100nM demonstrating an 11-fold increase in expression at 24 hours (p=0.19) relative to untreated control, which a 13-fold increase in expression was observed after exposure to 250nM doses after 8 hours (p=0.13) and 24 hours (p=0.23) relative to untreated control (**Figure 4.6**). These increases in AT1R expression appeared transient however, with expression levels reducing after 48 hours until experiment completion at 96 hours.

Increases in AT1R gene expression relative to control were also measured at doxorubicin doses of 50nM and 500nM, however the differences in these expression levels were lower compared to 100nM and 250nM, and did not achieve statistical significance (p>0.05). In contrast, despite doses of 500nM increasing AT1R expression up to 6-fold, these expression levels were lower than those observed in the 100nM and 250nM groups, thereby proposing that the cytotoxic effects of doxorubicin may be exerted upon the cardiomyocytes and therefore limit AT1R expression.

4.5 Effects of sub-therapeutic concentrations of doxorubicin on cellular expression of the AT1R protein

AC10 cardiomyocytes in plateau growth phase were exposed to clinically relevant doxorubicin concentrations (50nM, 100nM, 250nM, 500nM) and exposures (4-96 hours), with protein expression of AT1R determined by western blotting and normalised to expression of beta-actin (**Figure 4.7** and **Figure 4.8**).

As demonstrated in **Figure 4.7**, AT1R protein expression levels were detectable in the AC10 cardiomyocytes, with a consistent dose-related relationship observed at 8 hours but not with other time points after drug exposure (p=0.142). As would be expected, AC10 cardiomyocytes exposed to doxorubicin for only 4 hours demonstrated no significant increase in AT1R expression levels (p=0.24). This result remains consistent with gene expression levels, as the short exposure time appears insufficient to stimulate detectable increases in AT1R gene expression and subsequent protein synthesis.

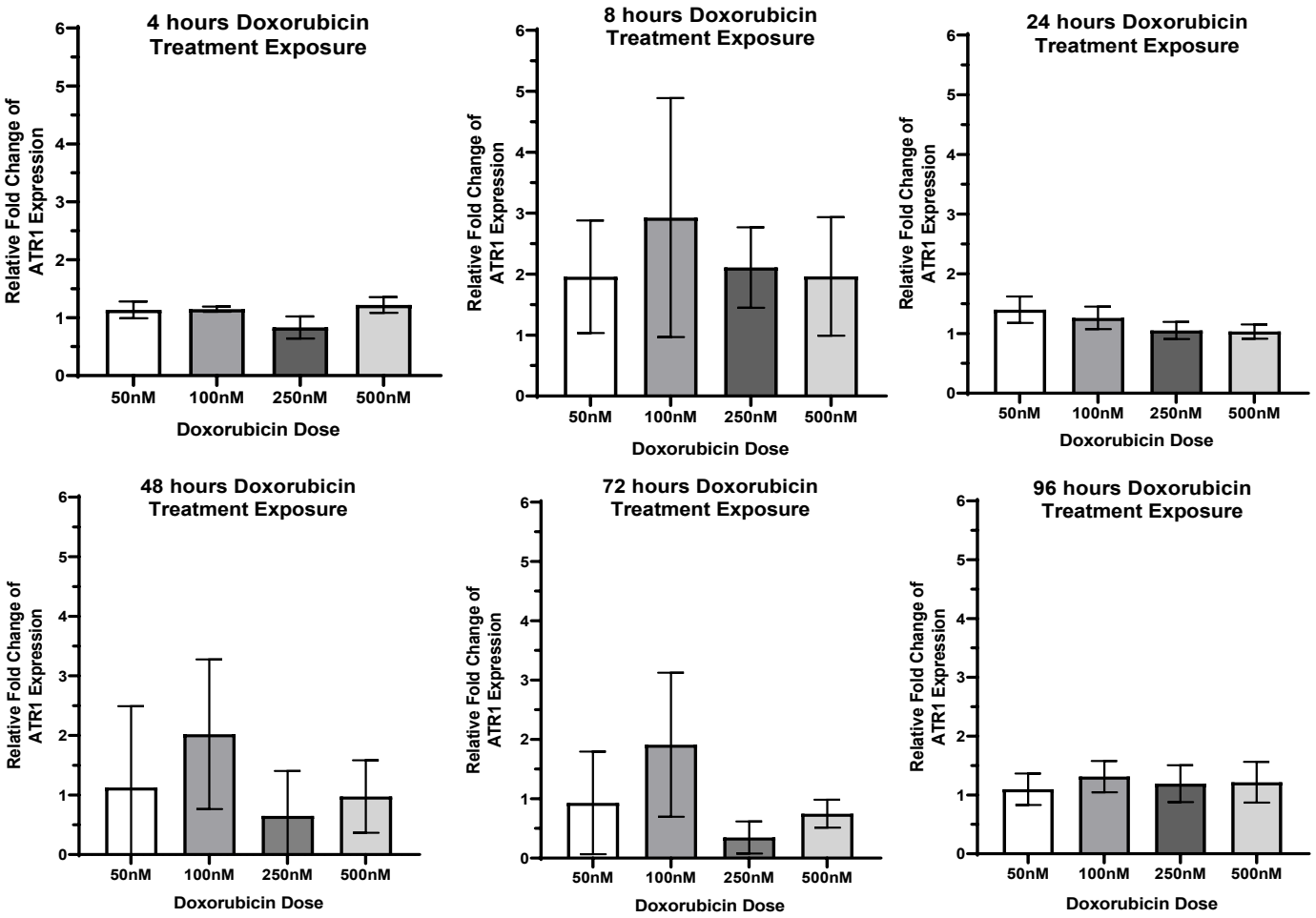


Figure 4.7 **Effect of doxorubicin dose exposure upon AT1R protein expression within *in vitro* AC10 cells.** AC10 cardiomyocytes were exposed to doxorubicin at a range of sub-toxic concentrations, across clinically relevant time points. AT1R protein expression determined by Western blotting, with expression changes calculated relative to time-matched vehicle control, after normalisation to beta-actin. Results are derived from the mean of at least three experiments (+/- SE). Statistical analysis was completed using ANOVA.

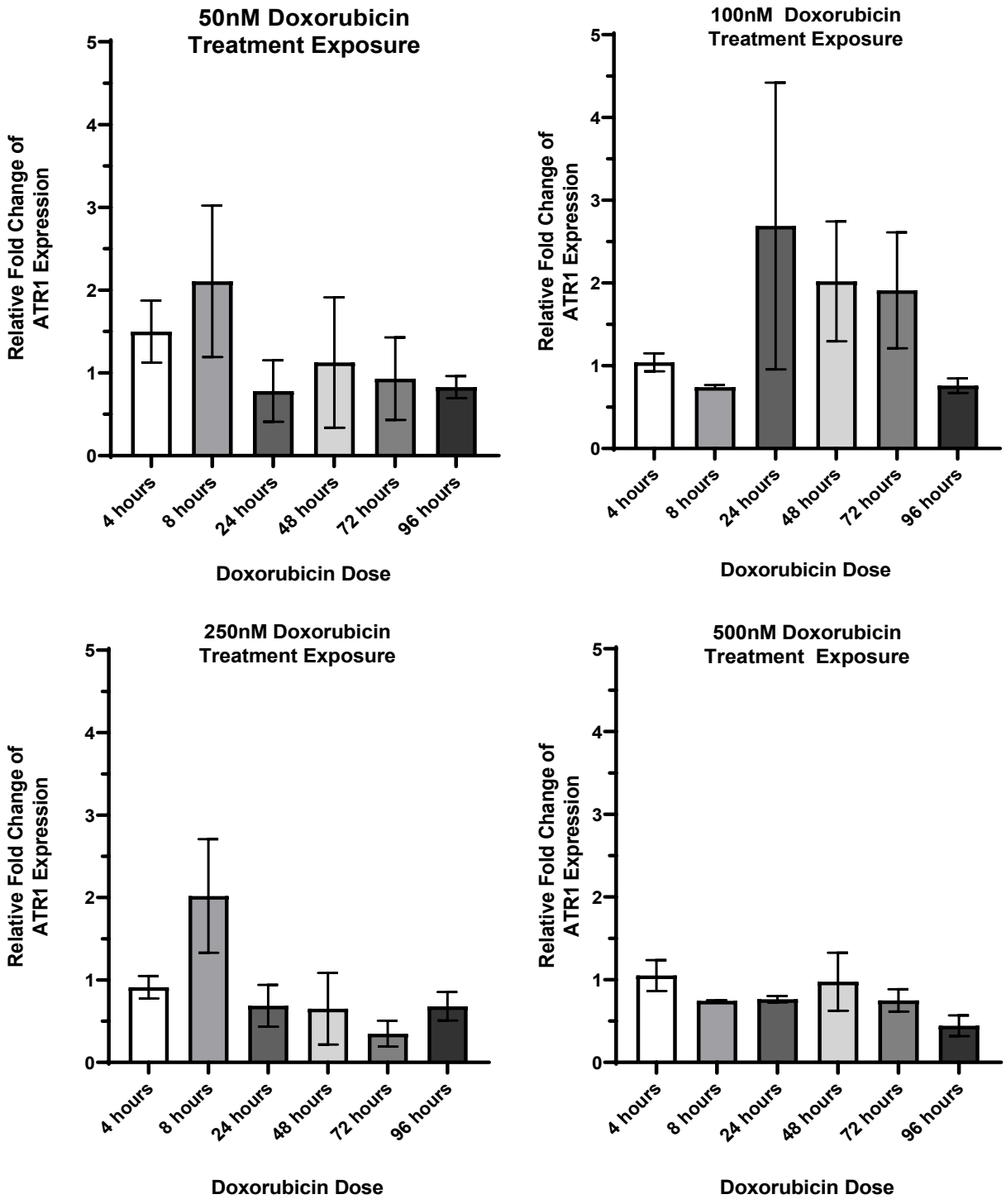


Figure 4.8 **Effect of doxorubicin exposure duration upon AT1R protein expression within *in vitro* AC10 cells relative to vehicle control.** AC10 cardiomyocytes in plateau growth phase were exposed to sub-toxic doxorubicin concentrations (0-500nM) for the clinically relevant time points of 4, 8, 24, 48, 72 and 96 hours. Protein expression of AT1R was determined by western blotting, normalised to expression of beta-actin and calculated relative to dose-matched vehicle control. Results are derived from at least three experimental repeats (+/- SE). Statistical analysis was completed using ANOVA.

A time dependent trend was observed at all concentrations (**Figure 4.8**) (P=0.361), with greatest increases in AT1R expression observed with cardiomyocytes treated with doses of 100nM doxorubicin. At this concentration, protein expression demonstrated a 2.7-fold increase (p=0.95) after 24 hours doxorubicin exposure, with a 2.0-fold increase still measurable after 48 hours (p=0.93) and at 72 hours (p=0.75). By 96 hours, the AT1R protein expression levels had returned to similar levels as the untreated control.

4.6 Effects of sub-therapeutic concentrations of doxorubicin on cellular expression of the AT1R, determined by antibody-mediated immunofluorescence staining

AC10 cardiomyocytes in plateau growth were exposed to clinically relevant concentrations of doxorubicin for 24 hours, with AT1R expression determined by immunofluorescence.

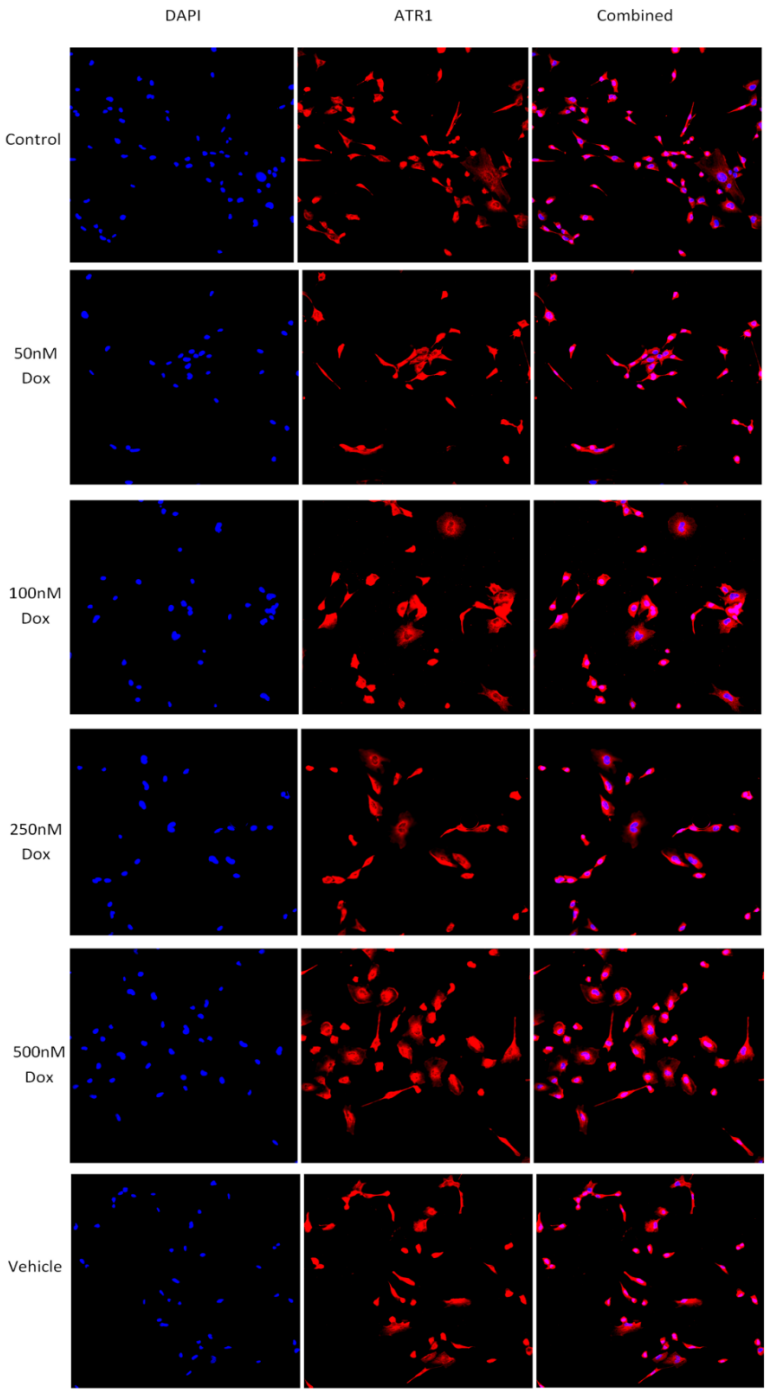


Figure 4.9 **Immunofluorescence imaging of *in vitro* AC10 cells stained for AT1R expression following 24 hours doxorubicin exposure.** Primary antibody AT1R 1:200. Secondary antibody 1:400. 35% magnification. AC10 cardiomyocytes were seeded at a density of $7.5x10^4$ cells/well and cultured until plateau achieved. Cells were fixed and stained for AT1R expression (red) and compared with vehicle control, whilst the nuclei were stained with DAPI (blue). AC10 cardiomyocytes were exposed to a series of sub-toxic concentrations of doxorubicin. When compared with control and vehicle control, AC10 cardiomyocytes treated with doxorubicin demonstrated increased AT1R expression, with a dose-dependent relationship observed.

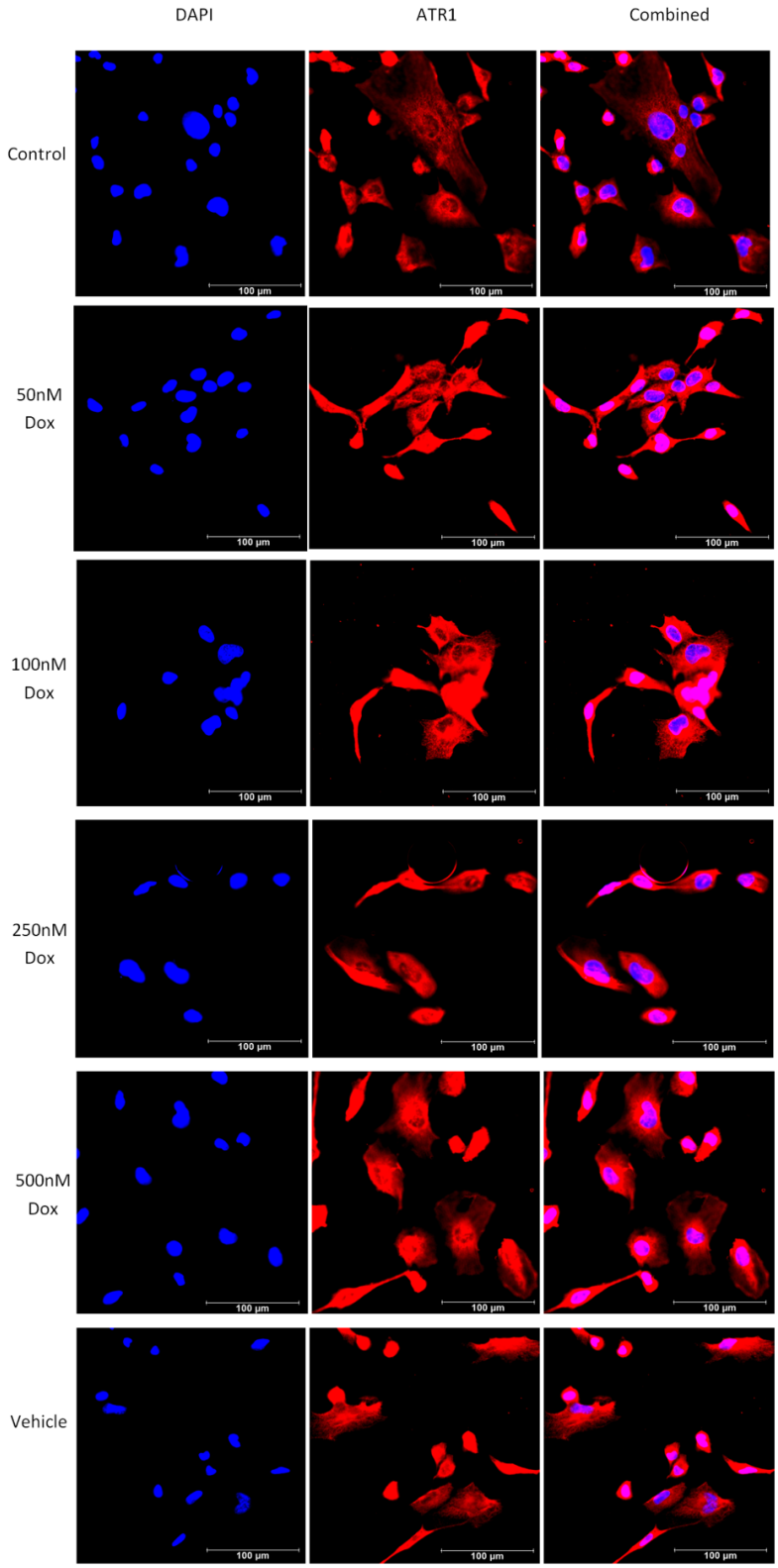


Figure 4.10 **Immunofluorescence imaging AT1R expression in AC10 cardiomyocytes after 24 hours doxorubicin exposure.** Primary antibody AT1R 1:200. Secondary antibody 1:400. 100% magnification. Results again illustrating a dose-dependent relationship between doxorubicin dose exposure and AT1R expression. Scale bars 100µm.

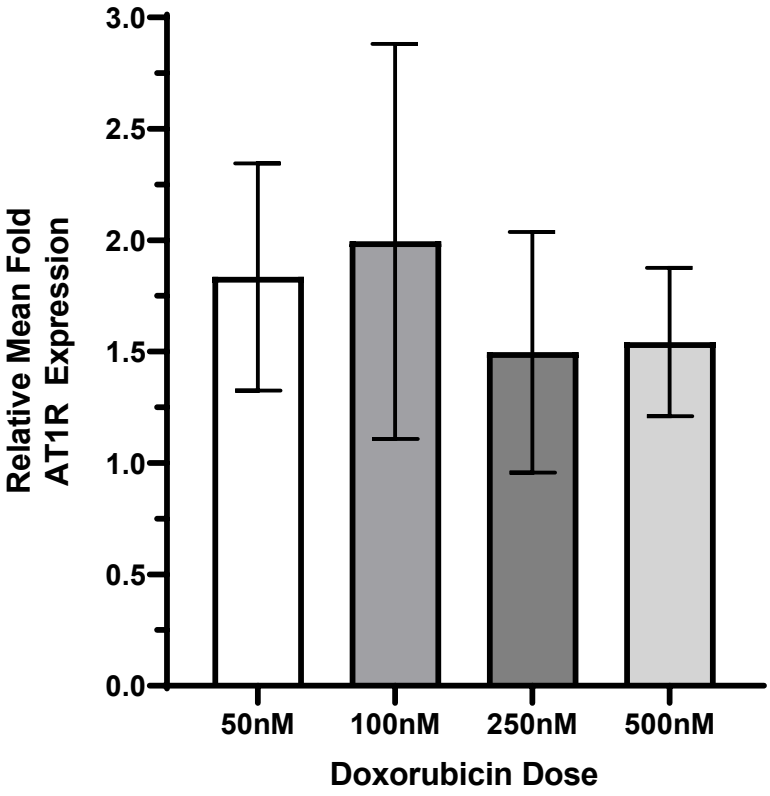


Figure 4.11 **Overall immunofluorescence image intensity of AT1R expression in AC10 cardiomyocytes exposed to 24 hours doxorubicin relative to vehicle control** (± s.d., n=3). Statistical analysis was completed with the independent samples t-test.

After background fluorescence subtraction, all doxorubicin concentrations produced an increase in AT1R expression relative to vehicle control, with a maximal 2-fold increase observed at concentrations of 100nM (p=0.378) (**Figure 4.11**). The drug vehicle itself did not induce a significant change in expression of AT1R (p=0.185; data not shown).

Analysis of the mean intensity distribution of AT1R expression across the cells was calculated by measuring the spatial distribution of intensities within each cardiomyocyte (see example **Figure 4.12**). Higher density of AT1R expression were detected in the main body of the cell relative to the periphery, indicating reduced expression at sites of cell-cell interactions compared to the apical cell surface (see appendix D).

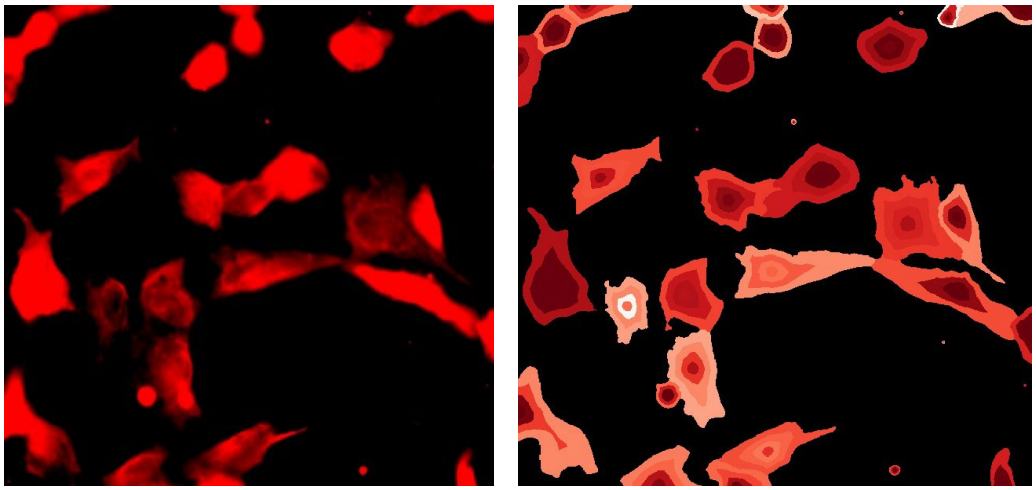


Figure 4.12 Example of cellular AT1R expression distribution within AC10 cardiomyocytes exposed to 250nM doxorubicin for 24 hours

4.6.1 Effect of doxorubicin upon angiotensin II concentrations of AC10 cardiomyocytes in vitro

Angiotensin II concentrations were measured within the media of AC10 cardiomyocytes treated with sub-therapeutic doxorubicin doses (**Table 4.4**). Angiotensin II was detectable in the culture media of AC10-CM cells, but not in the absence of cells, indicating production and secretion by the human cells. No significant increase in angiotensin II levels in cell culture media were detected following exposure of AC10-CMs to clinically relevant concentrations doxorubicin. In the presence of 500nM doxorubicin, levels of detectable angiotensin II were reduced relative to vehicle control treated cells, a likely reflection of cytotoxicity and cell loss under these conditions. Taken together this supports a lack of stimulation of angiotensin II production and secretion by AC10-CMs in response to doxorubicin induced stress and signalling pathways.

Table 4.4 Angiotensin II concentrations measured in media from AC10 cardiomyocytes treated with doxorubicin

Treatment	Angiotensin (pg/mL)
Control (No drug)	23.3 ± 15.8
100nM	22 ± 11.3
250nM	23.5 ± 8.6
500nM	15.9 ± 6.0
Media alone (no cells)	<3.7

4.6.2 Optimisation of assay to determine angiotensin II concentrations in human blood plasma

Plasma angiotensin II concentrations were initially measured in the venous blood of 10 healthy volunteers, with the mean concentration calculated as 32.3 pg/mL and mean concentrations ranging from 14.3 to 113.9 pg/mL (**Table 4.5**).

Table 4.5 Angiotensin II levels in blood plasma of healthy volunteer subjects. Angiotensin II concentrations were measured by ELISA, with data presented as mean ± SD of at least three independent experiments.

Healthy Volunteer	Angiotensin II Concentration (pg/mL) Mean ± SD	Gender
1	54.4 ± 30.5	Male
2	22.2 ± 14.7	Female
3	28.9 ± 22.9	Male
4	21.0 ± 16.5	Female
5	113.9 ± 39.9	Male
6	63.3 ± 30.9	Male
7	17.5 ± 11.0	Female
8	37.0 ± 24.6	Female
9	14.3 ± 8.4	Female
10	31.9 ± 3.1	Male
Mean	**32.3 ± 16.8**	**-**

Plasma from males (n=5) displayed a higher mean angiotensin II concentration compared to females (n=5), (58.5 ± 34.2 vs 22.4 ± 8.7 pg/mL), which was found to be statistically significant (p=0.03). Angiotensin II concentrations were also analysed across age groups (**Figure 4.13**), with the younger healthy volunteers demonstrating a higher mean concentration (46.3 ± 34.8 pg/mL) compared to older healthy volunteers (26.6 ± 8.9 pg/mL) (p=0.20). However, it is important to note that the older patients in the cohort were females, and females demonstrated a lower mean angiotensin II concentration compared to males.

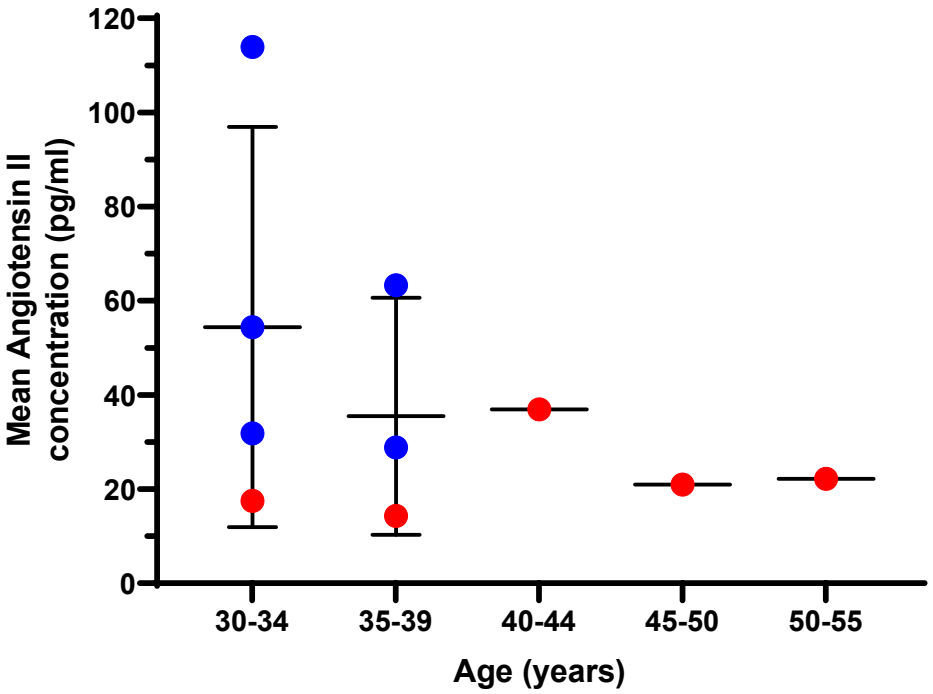


Figure 4.13 **Mean angiotensin II plasma concentrations in healthy volunteers grouped according to age.** Male healthy volunteers are signified by blue dots, whilst females are coloured in red. Data is represented as mean ± S.D.

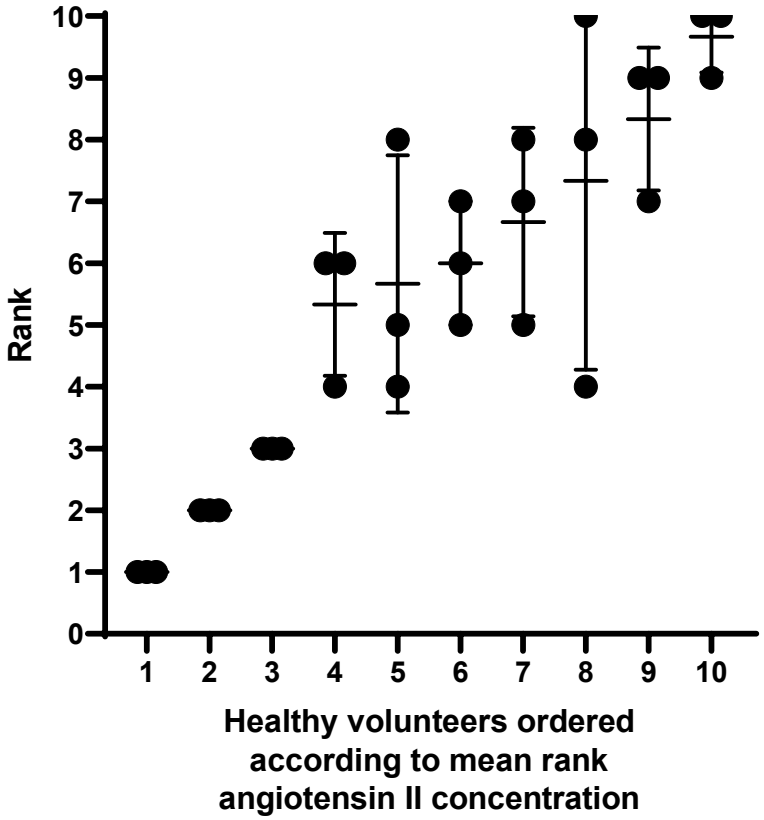


Figure 4.14 **Healthy volunteers ranked according to mean rank angiotensin II concentration.** The plasma angiotensin II concentrations were measured across 10 healthy volunteers, with volunteers then ranked according to angiotensin II concentration (1 = highest concentration, 10 = lowest). This process was repeated (n=3) to evaluate any change in rank between the experiments to provide an indicator of variability and sample stability. Error bars indicate variation in the rank between experiments, with an absence of error bars indicating no change in rank.

Angiotensin II concentrations from each experiment were ranked, with 1 representing the highest concentration and 10 the lowest, across three experimental repeats (**Figure 4.14**) Healthy volunteers with the highest angiotensin II concentrations demonstrated the least variability in rank, whilst patients with low to intermediate angiotensin II concentrations displayed the greatest variation. Overall, these results show that within a small sample of healthy volunteers, whilst there is variation between individual experiments (as indicated by the standard deviations in **Table 4.5**) and intra-individual variability, the mean values remain in order indicating little variation in angiotensin II concentration ranks and thus the assay is reliable.

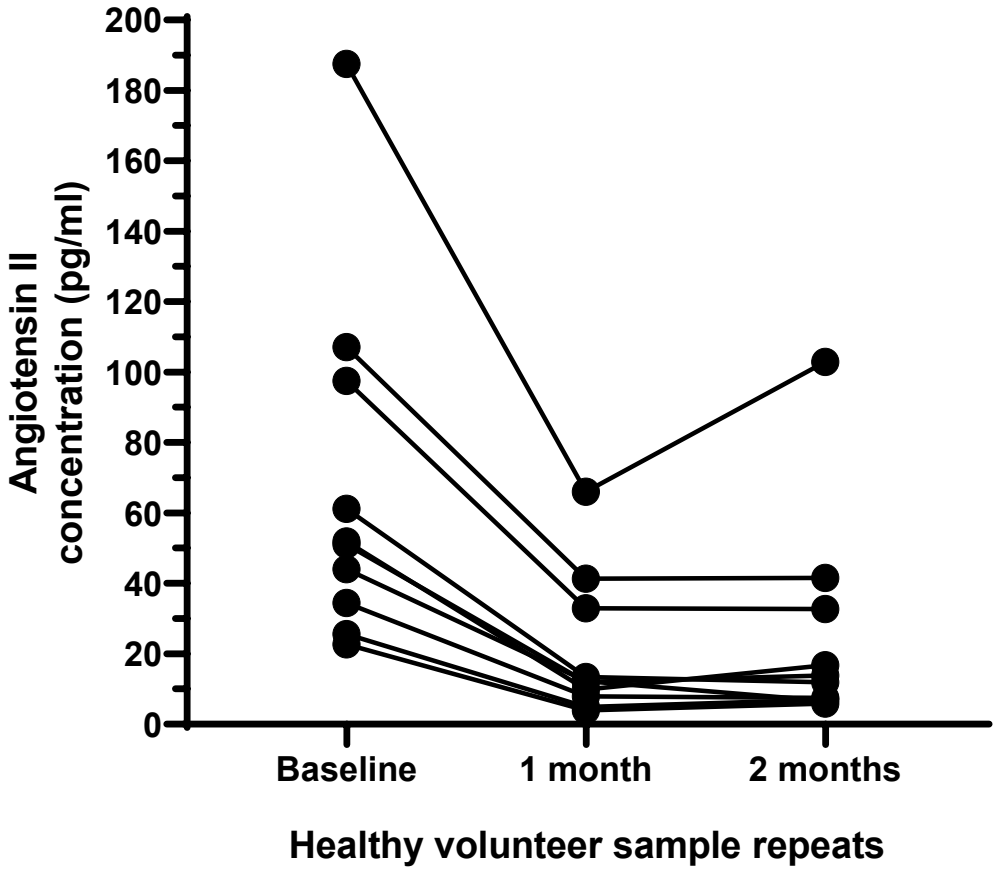


Figure 4.15 **Evaluation of the effects of plasma sample storage upon measured angiotensin II concentrations.** Healthy volunteer samples were evaluated across 3 freeze thaw cycles to evaluate the effects of storage upon angiotensin II concentrations and sample degradation.

The influence of sample storage upon mean angiotensin II measurements was also evaluated in the healthy volunteer plasma samples, with the variations in plasma angiotensin II levels calculated across multiple freeze thaw cycles and illustrated in **Figure 4.15**. The mean angiotensin absorbance readings across the freeze-thaw cycles demonstrated effects from sample freezing (**Figure 4.16**). This analysis demonstrated within the first month from samples collection (i.e. baseline) to resampling, a statistically significant change in angiotensin II concentrations between freeze thaw cycles ($p<0.05$). Therefore, to ensure accuracy of plasma angiotensin II measurements, samples should be analysed from the time of sampling to ensure no degradation in angiotensin II concentration.

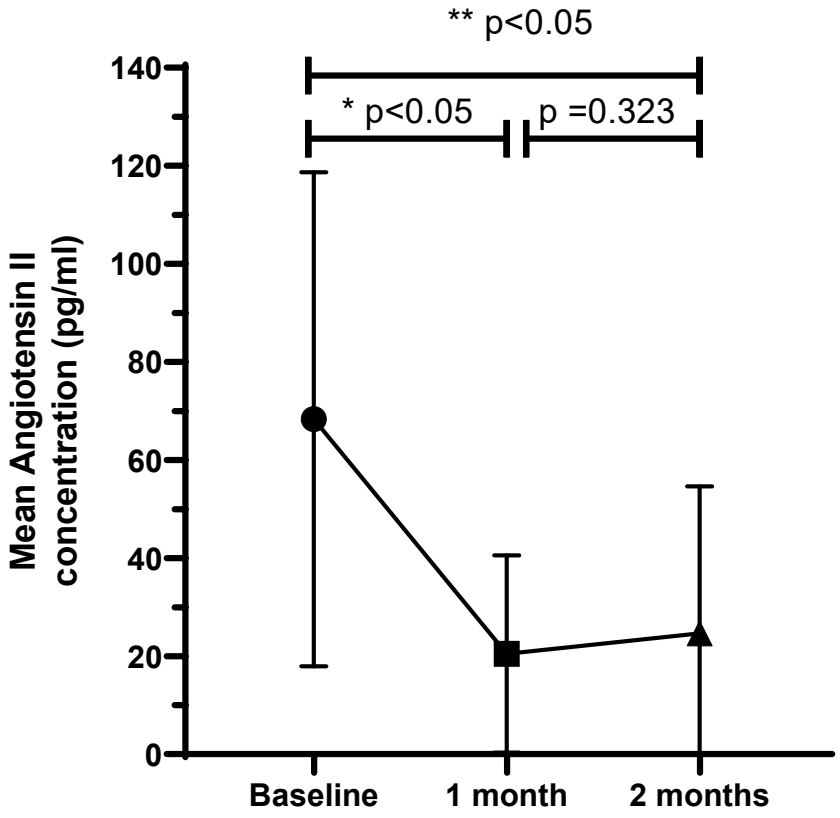


Figure 4.16 Analysis of the influence of freeze-thaw cycles upon mean plasma angiotensin II concentrations within healthy volunteer samples. Mauchly's test of sphericity was assumed during statistical analysis, with p-values calculated from the Bonferroni test.

The role of protease inhibitors for angiotensin II stabilisation was independently analysed, with a protease inhibitor cocktail added to venous blood samples prior to plasma isolation and sample storage. No significant difference in angiotensin II concentration was identified between the treated and untreated samples.

4.6.3 Relationship between ACE genotype and plasma angiotensin II concentrations in normal population

None of the healthy volunteers displayed pre-existing cardiac conditions or were receiving any cardiac medications at the time of receipt of samples. DNA was successfully extracted from buccal swabs of 9 of the 10 healthy volunteers. ACE genotyping revealed 55.6% (5/9) of healthy volunteers displayed the homozygous D/D genotype, whilst 44.4% (4/9) were genotyped as the heterozygous I/D (**Figure 4.17**). No samples exhibited the homozygous I/I genotype.

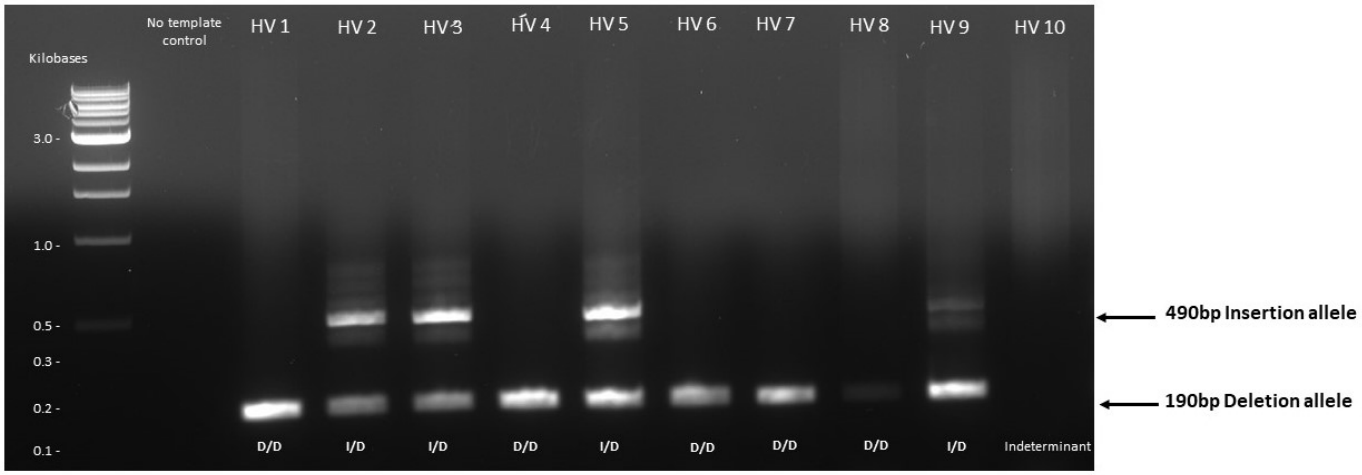


Figure 4.17 **ACE genotypes in healthy volunteers as determined by Rigat et al. (1992) primers.** Reverse transcriptase PCR demonstrating expression of ACE genotypes in cDNA generated from healthy volunteers. Expected band size for insertion allele 490bp, and 190bp for the deletion allele. The molecular markers (kilobases) for determining product size and indicated on the left.

All samples initially identified as homozygous deletion (D/D) were re-evaluated using Insertion-specific primers (Biller et al., 2006), to confirm the D/D genotype, with all samples confirmed as being identified correctly as D/D with no mistyping (**Figure 4.18**). The distribution of the D/D, I/D and I/I genotypes were not consistent with those predicted by the Hardy-Weinberg equilibrium (genotype frequencies 0.44, 0.44, 0.11 respectively).

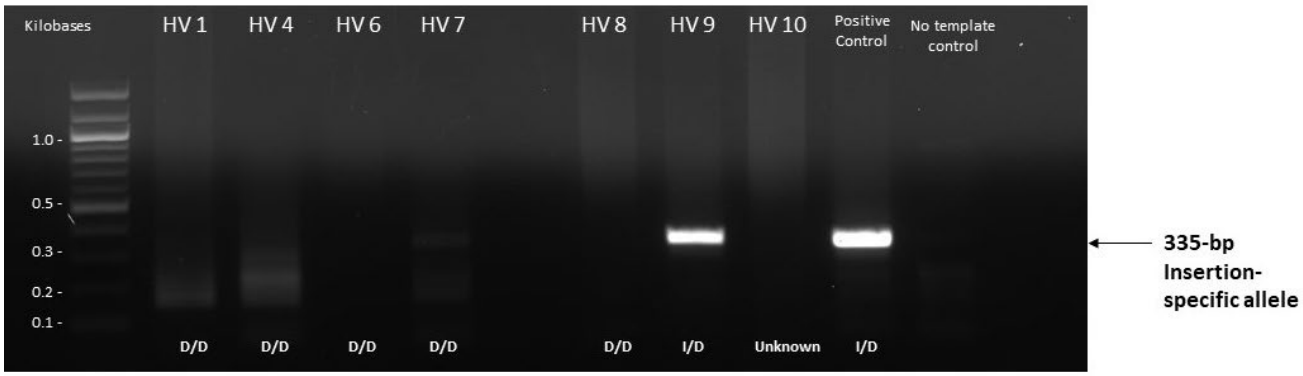


Figure 4.18 **Insertion specific ACE primer analysis of healthy volunteers.** Due to preferential amplification of Deletion allele in ID heterozygotes, these subjects can be mistyped as having the D/D genotype instead of I/D. Reverse transcriptase PCR was again used to re-evaluate D/D homozygous genotyped healthy volunteers. The agarose gel electrophoresis demonstrated that all D/D healthy volunteers were correctly typed, as confirmed by the absence of the insertion-specific primer at 335bp.

Table 4.6 **Relationship of mean angiotensin II concentration and ACE genotype within healthy volunteers.** Mean angiotensin II concentrations as measured by ELISA (±S.D.), and ACE genotypes determined from Rigat et al. (1992) primers within health volunteers. It was not possible to determine the ACE genotype in one healthy volunteer.

Healthy Volunteer	Angiotensin II Concentration (pg/mL) Mean ± SD	ACE Genotype
1	54.4 ± 30.5	D/D
2	22.2 ± 14.7	I/D
3	28.9 ± 22.9	I/D
4	21.0 ± 16.5	D/D
5	113.9 ± 39.9	I/D
6	63.3 ± 30.9	D/D
7	17.5 ± 11.0	D/D
8	37.0 ± 24.6	D/D
9	14.3 ± 8.4	I/D
10	31.9 ± 3.1	Indeterminant

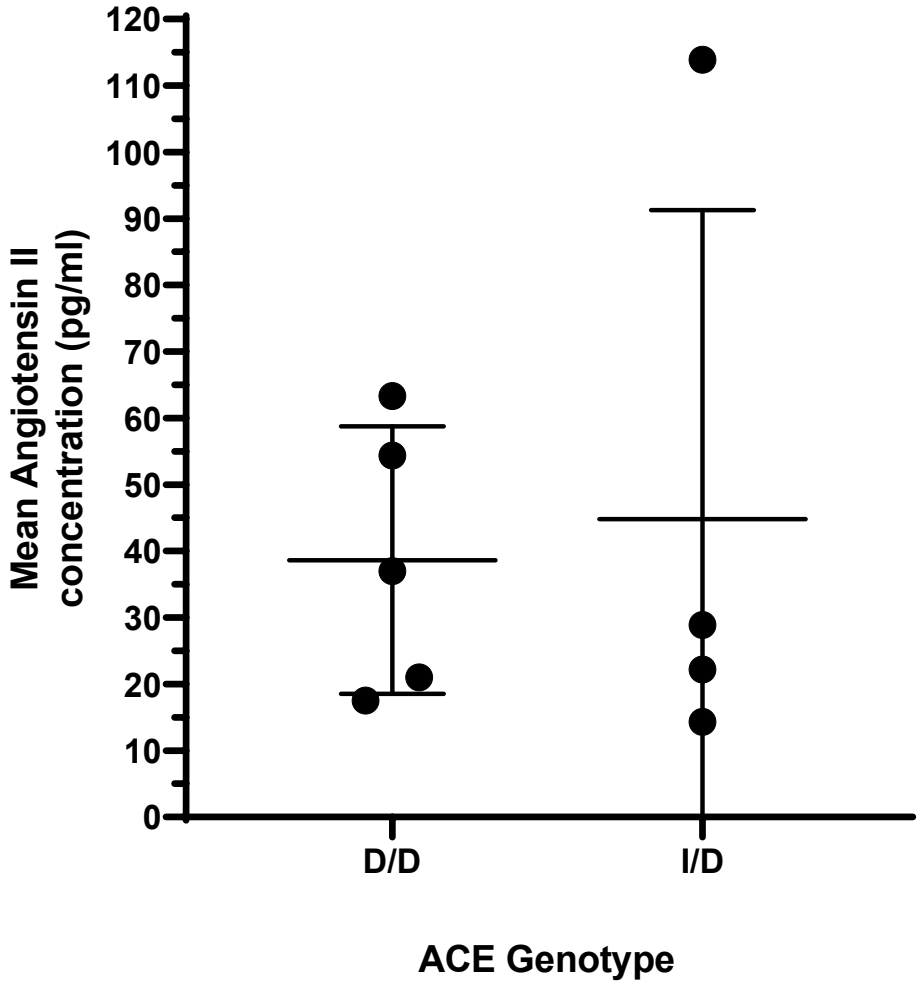


Figure 4.19 **Mean angiotensin II concentrations grouped according to ACE genotype.** Mean angiotensin II concentrations for each healthy volunteer as determined by ELISA, grouped according to ACE genotype as established by Rigat et al. (1992) primers. Values represented as mean ± S.D.

Evaluating angiotensin II levels based upon ACE genotype (**Figure 4.19**) demonstrates similar mean angiotensin II concentrations between the D/D (38.6 ± 20.1 pg/mL) and I/D genotypes (44.8 ± 46.4 pg/mL), with no statistical difference between the two means (p-value =0.816). Within the I/D genotype group, the 113.9 pg/mL value is much greater than the other mean angiotensin II measurements, and therefore strongly influences the mean and standard deviation of this the I/D group.

4.6.4 Clinical characteristics of patients diagnosed with the cardiotoxicity phenotype following treatment with anthracycline chemotherapy for breast cancer

A total of 17 patients (mean age 58.8 ± 8.7 years, 100% females, 100% breast cancer) who underwent echocardiography after starting chemotherapy were included in the study, with available data to determined cardiotoxicity status, and access to plasma samples for angiotensin and DNA analysis. The median follow-up following chemotherapy completion was 11.4 years (interquartile range, 10.3 – 13.5 years; range 9 to 15 years). All patients (100%) were followed up for greater than 5 years, whilst 88% were followed-up for more than 10 years since completion of treatment. On last contact, 5 patients (29%) had died (3 breast cancer (60%) and 2 (40%) due to other causes), no patients died from cardiovascular disease.

Cardiotoxicity was identified in 10 patients (58.8%), whilst 7 patients (41.2%) had no evidence of cardiotoxicity on echocardiogram. The characteristics of these patients with and without cardiotoxicity are shown in **Table 4.7**.

From first dose of anthracycline chemotherapy to cardiotoxicity diagnosis, the mean time was 26.3 months with a median of 12.5 months (interquartile range, 7 – 45 months). From the last dose of anthracycline chemotherapy to cardiotoxicity diagnosis, the mean time was 23.9 months, and the median was 10 months (interquartile range, 4 – 43 months). **Figure 4.20** illustrates the cumulative incidence of cardiotoxicity from the last dose of anthracycline chemotherapy. Seven cases (70%) of cardiotoxicity were diagnosed within the first 12 months of completing chemotherapy, with three cases (30%) diagnosed after one year.

Heart failure therapy was initiated in 60% of patients diagnosed with cardiotoxicity, with patients receiving both an ACE inhibitor and a beta-blocker, titrated to maximal tolerated doses. There were no recorded cardiovascular or cardiotoxicity-related deaths within the patient cohort.

Table 4.7 Clinical characteristics of patients within the anthracycline pharmacogenetics cohort developing or not developing anthracycline-induced cardiotoxicity. Data are expressed as numbers (%) or mean±SD. AC indicates doxorubicin, cyclophosphamide; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; EC, epirubicin, cyclophosphamide; FAC, 5-fluorouracil, doxorubicin, cyclophosphamide. LVEF, left ventricular ejection fraction; EGFR, Estimated glomerular filtration rate,; SD, standard deviation.* Cumulative anthracycline dose was calculated by converting the anthracycline agents into doxorubicin equivalents. $Calculated by Cockcroft-Gault formula; 10-year cardiovascular risk estimated using QRISK®3 (https://www.qrisk.org)

Variable	Cardiotoxicity (n = 10)	No Cardiotoxicity (n = 7)	P-value
Age at time of starting chemotherapy, y (mean ±SD)	60.9±7.3	55.7±10.0	0.236
Age at time of completing chemotherapy, y (mean ±SD)	72±7	67±11	0.224
Cumulative anthracycline dose, mg/m^2 *	372±76	395±54	0.794
LVEF on echo, n (%): - **Mild (46-54%)** - **Moderate (36-45%)** - **Severe (≤ 35%)**	 9 (90%) 1 (10%) -	 - - -	
Hypertension, n (%)	4 (40%)	1 (14.3%)	0.278
Diabetes mellitus, n (%)	2 (20%)	1 (14.3%)	0.640
Hypercholesterolaemia, n (%)	1 (10%)	1 (14.3%)	0.669
Ischaemic heart disease, n (%)	0 (0%)	1 (14.3%)	0.412
Atrial fibrillation, n (%)	2 (20%)	0 (0%)	0.485
Body mass index, kg/m^2 (mean ±SD)	29.9±8.1	29.2±5.9	0.860
EGFR (mL/min/1.73m^2) (mean ±SD) $	78±14	79±10	0.856
Oncology schedule, n (%): - **AC** - **FEC** - **EC**	 6 (60%) 2 (20%) 2 (20%)	 2 (28.6%) 5 (71.4%) -	0.218 -
Adjuvant Herceptin, n (%)	9 (90%)	4 (57.1%)	0.162
Mediastinal radiotherapy, n (%)	8 (80%)	5 (71.4%)	0.559
ACEi or ARB therapy, n (%)	6 (60%)	3 (42.9%)	0.419
Beta-Blocker therapy, n (%)	6 (60%)	1 (14.3%)	0.08
Statin therapy	4 (40%)	6 (85.7%)	0.08
10-year Cardiovascular risk score (Q-Risk) mean ±SD (%) ¶	14.6±5.6	20.7±11.1	0.197
Mortality at time of analysis, n (%): - **Breast cancer** - **Cardiovascular** - **Other**	 1 (10%) - 1 (10%)	 2 (28.6%) - 1 (14.3%)	0.309 0.360 0.669

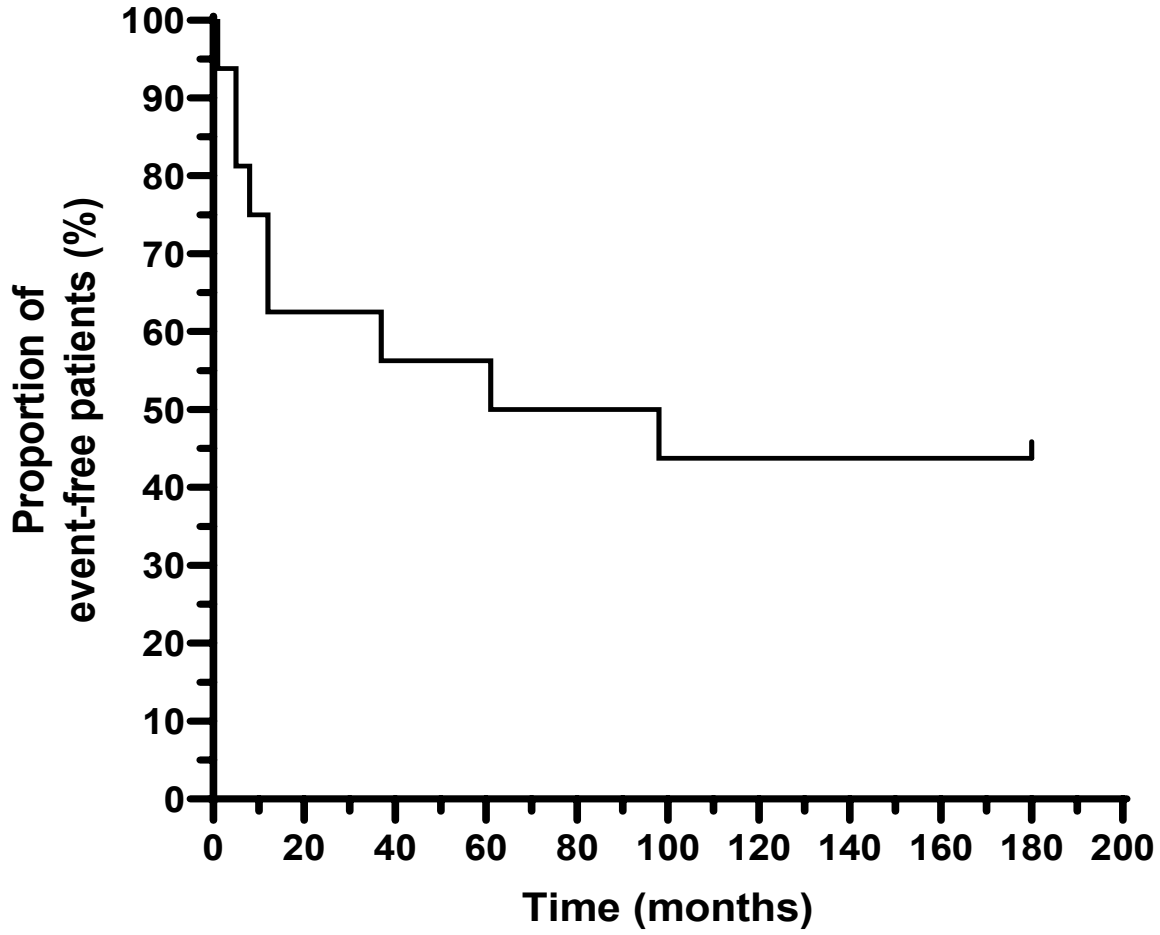


Figure 4.20 Kaplan-Meier curve demonstrating the cumulative incidence of anthracycline-induced cardiotoxicity in patients treated for breast cancer within the ACPK Study

Statistical analysis for the patient characteristics was completed, with no statistical differences calculated between the cardiotoxicity and non-cardiotoxicity patient groups. There was no statistical difference between the cardiovascular risk scores of patients diagnosed with cardiotoxicity and without cardiotoxicity.

4.6.5 Plasma angiotensin II concentrations in breast cancer patients treated with anthracycline chemotherapy according to long-term cardiotoxicity status

Plasma angiotensin II concentrations were measured in 28 patients from the anthracycline pharmacogenomics breast cancer clinical trial patient cohort (**Table 4.8** and **Table 4.9**), together with cardiotoxicity statuses and ACE genotype. The mean angiotensin II concentration across the patient cohort was 57.1 ± 40.4 pg/mL, (ranging from 10.1 to 171.3 pg/mL) (**Table 4.8, Table 4.9, Figure 4.22)**

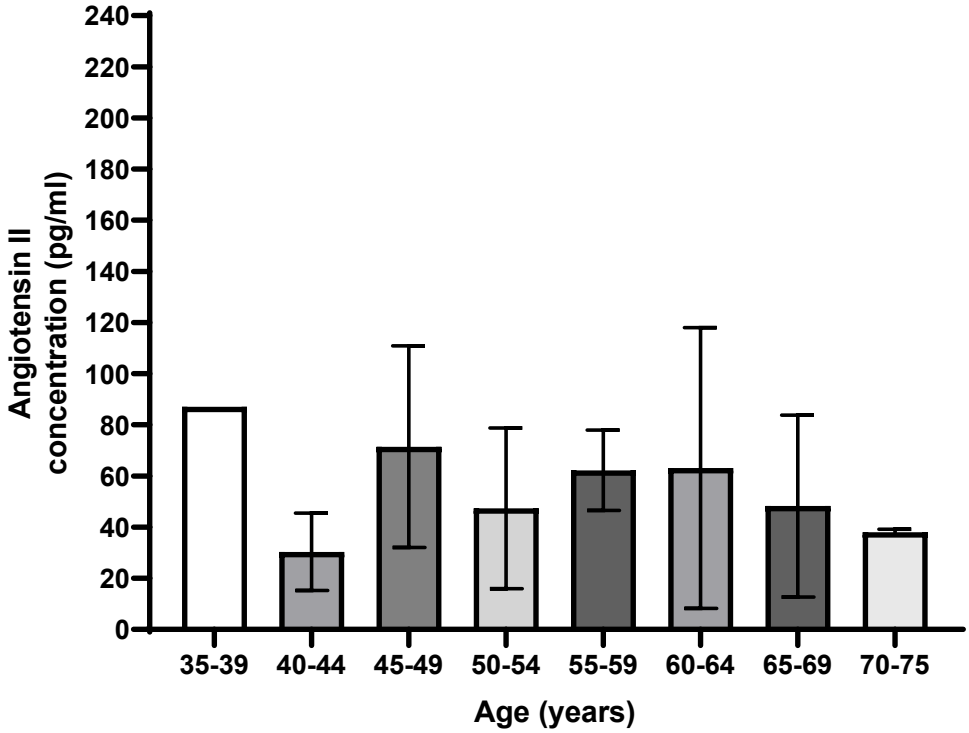


Figure 4.21 Analysis of mean angiotensin II concentrations within the patient cohort grouped according to age. Error bars indicative of S.D.

Of the 17 patients with known cardiotoxicity status as diagnosed by echocardiogram, angiotensin II concentrations were identified for 15 patients, of which 8 had confirmed cardiotoxicity (53.3%) and 7 had no evidence of cardiotoxicity (46.7%) (**Table 4.7**). None of these patients were prescribed ACE inhibitors or angiotensin receptor blockers (ARBs) prior to chemotherapy treatment and blood sampling, removing this as a potential confounding factor within the study. Analyses of plasma angiotensin II concentrations according to patient age at time of receiving chemotherapy indicated no significant relationship between patient age and angiotensin II levels (**Figure 4.21**). However, the mean plasma angiotensin levels in this patient cohort (73.4 ± 46.4 pg/mL) were significantly higher than the levels determined in the healthy individual female cohort (22.4 ± 8.7 pg/mL; Section 4.6.2) (p=0.001), although it is of note that the mean age between these groups was different at 58.8 ± 8.7 years compared to 41.0 ± 9.6 years (p=0.01), accordingly.

Evaluation of a relationship between pre-chemotherapy plasma angiotensin II levels and cardiotoxicity status showed a significant difference in mean plasma angiotensin II concentrations between the cardiotoxicity and non-cardiotoxicity cohorts (59.4± 33.0 vs. 89.4± 56.6pg/mL, p=0.245) (**Figure 4.23**).

Table 4.8 **Blood plasma angiotensin II levels, and ACE genotype in breast cancer patients treated with anthracyclines with known presence/absence of cardiotoxicity**

Patient	Angiotensin II Concentration (pg/mL)	Cardiotoxicity Status	ACE Genotype
ACPK 01	52.0	Yes	I/D
ACPK 02	67.2	No	I/D
ACPK 04	90.0	Yes	I/D
ACPK 11	58.9	No	D/D
ACPK 12	29.0	Yes	D/D
ACPK 13	19.7	Yes	I/D
ACPK 14	28.9	Yes	I/D
ACPK 18	110.0	Yes	D/D
ACPK 22	55.3	No	D/D
ACPK 25	171.3	No	D/D
ACPK 31	61.2	Yes	I/D
ACPK 41	26.0	No	I/D
ACPK 42	165.7	No	I/D
ACPK 44	84.5	Yes	I/I
ACPK 47	81.2	No	I/I
Mean	**73.4 ± 46.4**	**-**	**-**

Table 4.9 **Blood plasma angiotensin II levels, and ACE genotype in breast cancer patients treated with anthracyclines for which presence/absence of cardiotoxicity is unknown.**

Patient	Angiotensin II Concentration (pg/mL) Mean ± S.D.	Cardiotoxicity Status	ACE Genotype
ACPK 03	29.1	-	D/D
ACPK 05	55.1	-	D/D
ACPK 06	71.4	-	I/D
ACPK 07	15.8	-	I/D
ACPK 09	38.1	-	D/D
ACPK 10	41.0	-	D/D
ACPK 16	10.1	-	D/D
ACPK 17	45.4	-	D/D
ACPK 19	34.1	-	D/D
ACPK 20	24.8	-	D/D
ACPK 21	49.4	-	D/D
ACPK 23	87.0	-	D/D
ACPK 24	66.4	-	D/D
Mean	**43.7 ±21.4**		-

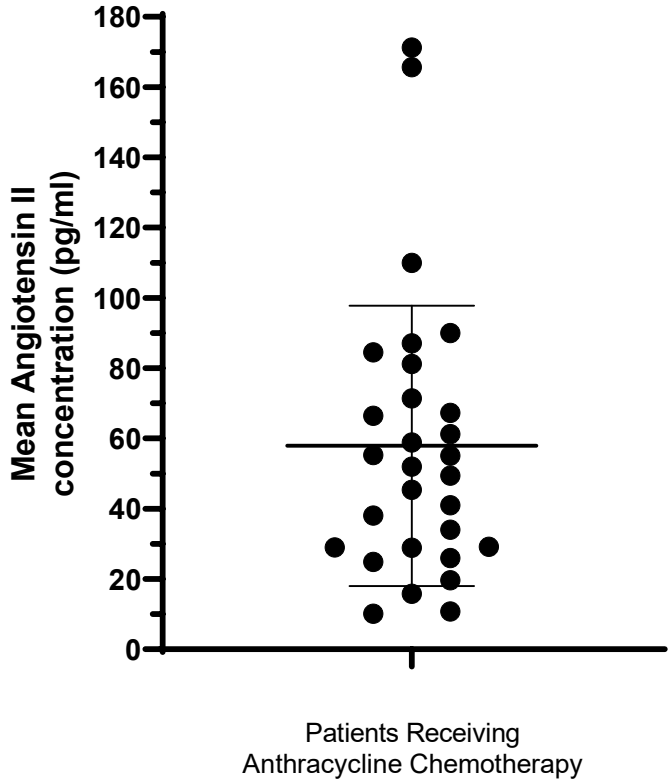


Figure 4.22 **Distribution of plasma angiotensin II concentrations in patients from the anthracycline pharmacogenomics breast cancer clinical trial cohort. (n=28)**

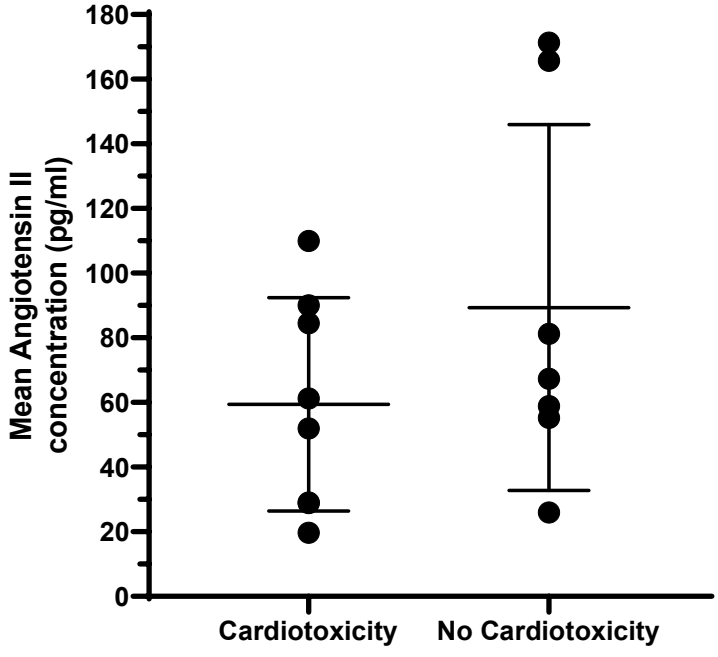


Figure 4.23 **Mean plasma angiotensin II concentrations within the patient cohort grouped according to cardiotoxicity status.** Data representative of mean concentration for each group with errors bars signifying standard deviation (n=15)**.**

4.6.6 ACE genotype and relationship to plasma angiotensin II levels in breast cancer patients treated with anthracycline chemotherapy.

The association between ACE genotyping and plasma angiotensin II concentration was also evaluated within the anthracycline pharmacogenomic cohort of 28 breast cancer patients, analysed according to genotype (**Table 4.9; Figure 4.24**). Patients with the I/I genotype (n=2) were shown to have the highest mean plasma levels of angiotensin II (82.9 ± 2.3 pg/mL) compared to patients identified as I/D genotype (59.8 ± 44.8 pg/mL; n=10), and D/D genotype (56.6 ± 39.2 pg/mL; n=16). The difference between the mean plasma angiotensin II concentrations of the D/D and I/I genotype was statistically significant (p=0.018). There was no statistically significant difference found between the D/D and I/D genotyped groups, or between the I/D and I/I groups.

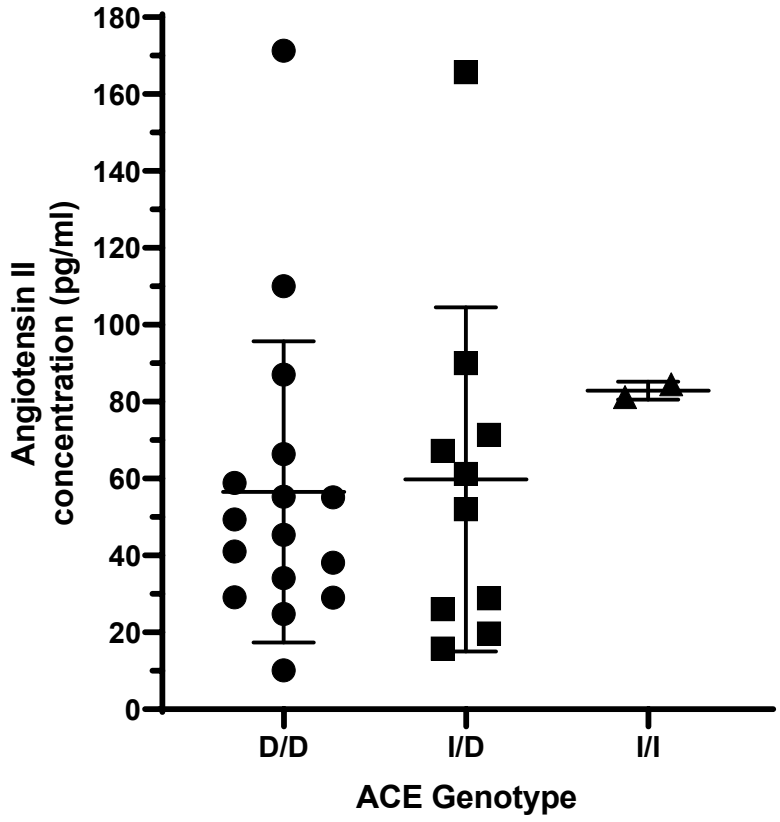


Figure 4.24 **Mean angiotensin II concentrations within the patient cohort grouped according to ACE genotype.** Data representative of mean concentration for each group with errors bars signifying standard deviation. D/D=deletion/deletion, I/D = insertion/deletion, I/I = insertion/insertion.

4.6.7 ACE genotype in breast cancer patients treated with anthracycline chemotherapy according to long-term cardiotoxicity status

ACE genotyping was also evaluated within the anthracycline pharmacogenomic cohort, with 15 patients selected for analysis based upon the confirmed presence (8 patients) or absence (7 patients) of the cardiotoxicity phenotype with echocardiography. This was complemented by analysis of a further 6 breast cancer patients with confirmed cardiotoxicity status, diagnosed and recruited from a cardiotoxicity clinic, (**Table 4.10**), with overall analyses performed for 14 patients with cardiotoxicity and 7 patients without cardiotoxicity. ACE genotype, including confirmation of potential insertion genotypes, identified D/D, I/D and I/I genotypes of 3:10:1 out of 14 patients (21%:71%:7%) in the cardiotoxicity positive cohort and 3:3:1 out of 7 patients (43%:43%:14%) in the cardiotoxicity negative cohort (**Table 4.10**). Acknowledging the number of samples in the analysis was low, an appreciable difference in genotype status between the two groups was not supported.

Table 4.10 **ACE genotypes of breast cancer patients treated with anthracycline chemotherapy according to long-term cardiotoxicity status.** 15 patients from the anthracycline pharmacogenomics cohort and 6 additional patients with breast cancer from the cardiotoxicity clinic were analysed. Patients were grouped according 14 patients were identified with the cardiotoxicity phenotype, whilst 7 patients were without the cardiotoxicity phenotype as diagnosed with echocardiography. ACE genotyping was determined by Rigat et al. primers (1992).

ACE Genotype	Cardiotoxicity (n=14)	No Cardiotoxicity (n=7)
D/D	3 (21.4%)	3 (42.9%)
I/D	10 (71.4%)	3 (42.9%)
I/I	1 (7.1%)	1 (14.3%)

ACE genotyping was also evaluated according to timing of cardiotoxicity diagnosis, with patients categorised according to acute (less than one year since completing chemotherapy) or chronic (more than one year after chemotherapy completion) (**Table 4.11**). No clear difference was observed between these two groups (both n=14) with D/D, I/D, I/I genotypes of 1:3:1 and 2:7:0, respectively.

Table 4.11 ACE genotyping in the anthracycline pharmacogenomics patient cohort evaluated against timing of cardiotoxicity diagnosis with echocardiogram

ACE Genotype	Acute: < 1 year until cardiotoxicity diagnosis (n=5)	Chronic: > 1 year until cardiotoxicity diagnosis (n=9)
D/D	1	2
I/D	3	7
I/I	1	0

4.6.8 ACE genotype and relationship to plasma angiotensin II levels in breast cancer patients treated with anthracycline chemotherapy according to long-term cardiotoxicity status

The association between ACE genotyping and plasma angiotensin II concentration was also evaluated within the anthracycline pharmacogenomic cohort of 15 breast cancer patients with diagnosed presence/absence of cardiotoxicity, analysed according to genotype (**Table 4.8; Figure 4.25**). Patients with the I/I genotype exhibiting cardiotoxicity (n= 1) had a mean angiotensin II concentration of 84.5 pg/mL, similar to patients without cardiotoxicity (n=1) of 81.2 pg/mL. In contrast, patients with the I/D genotype exhibiting cardiotoxicity (n=5) had a lower mean angiotensin II concentrations of 50.4 ± 27.8 pg/mL compared to patients without cardiotoxicity (n=3) who exhibited a mean plasma angiotensin concentration of 86.3 ± 71.8 pg/mL (p=0.48). In terms of the D/D genotype, patients with cardiotoxicity (n=2) showed mean plasma angiotensin levels of 69.5 ± 57.3 pg/mL, again lower than that detected in patients without cardiotoxicity (n=3) of 95.2 ± 66.0 pg/mL (p=0.68).

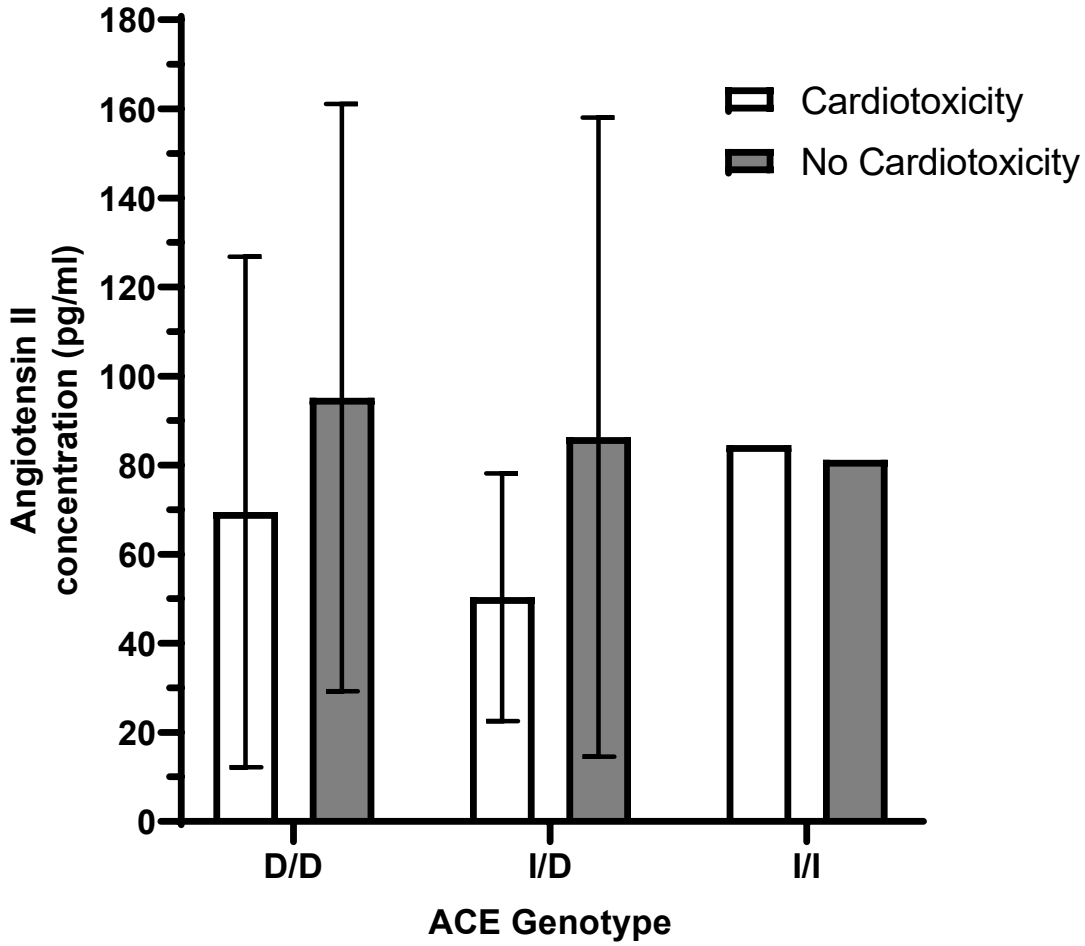


Figure 4.25 **Mean angiotensin II concentrations according to ACE genotype and cardiotoxicity status from 15 patients diagnosed with breast cancer and treated with anthracycline chemotherapy.** Results are represented as mean with errors bars indicating standard deviation.

4.7 Discussion

Anthracycline chemotherapy remains a key component of many cancer treatment regimens in both paediatric and adult patients. Despite their therapeutic efficacy, anthracyclines are associated with both acute and late-onset cardiac toxicities. Meta-analyses report an overt cardiotoxicity incidence of 6.3%, whilst sub-clinical cardiotoxicity incidence is 17.9% (Lotrionte et al., 2013). The major clinical complication of anthracycline-induced cardiotoxicity is the development of late-onset cardiotoxicity, occurring several years after drug administration, presenting as life-threatening heart failure (HF). Determining the relationship between subclinical anthracycline-induced cardiotoxicity and late-onset HF, strategies for mitigation of anthracycline-induced cardiotoxicity, and impacts upon the cancer survivor population remains a complex challenge.

One pathway that has gained increasing interest in the development of cardiomyopathies and is associated with regulation of several cardiac related physiological processes is the RAAS pathway. In the context of anthracycline-induced cardiotoxicity, a role for angiotensin signalling has been postulated. Several research groups have identified a relationship between angiotensin signalling and AIC, which can be separated into pre-clinical and clinical studies. Within pre-clinical research, Zheng et al. demonstrated a 3-fold increase in plasma and myocardial angiotensin II, after inducing cardiomyopathy with doxorubicin treatment in rats (Zheng et al., 2012). Upstream targets from angiotensin II have been analysed, with hamsters administered doxorubicin displaying a doubling of cardiac ACE activity relative to control. Furthermore, administration of ACE inhibitors in these hamsters, not only significantly reduced the levels of ACE activity, but also prevented myocardial hypertrophy and cardiac dysfunction (Okumura et al., 2002). Similarly, Rashikh et al. observed an increase in renin activity associated with anthracycline-induced cardiotoxicity, as rats treated again with doxorubicin demonstrated a near 5-fold increase in plasma angiotensin I levels, taken as a surrogate for renin activation (Rashikh et al., 2014). To evaluate the clinical impact of these findings, renin inhibition with aliskiren protected the rat hearts against myocardial injury from the doxorubicin therapy, with myocyte apoptosis, mitochondrial damage and nuclear membrane rupture all prevented by aliskiren, emphasising the link between RAAS signalling and anthracycline-induced cardiotoxicity. Crucially, downstream signalling effects within the RAAS caused by doxorubicin have also been identified, as angiotensin II receptor blockade

(AT1R), the principal binding site of angiotensin II, preventing doxorubicin-induced cardiac impairment (Toko et al., 2002).

Within the clinic, angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB) have been used to treat anthracycline-induced cardiotoxicity, and despite their efficacy being well studied for the treatment of heart failure, hypertension and post-acute coronary syndromes, their mechanism(s) for treating and preventing anthracycline-induced cardiotoxicity remain unknown (Cardinale et al., 2015, Vaduganathan et al., 2019, Heck et al., 2021).

It is thus hypothesised that if the adverse cardiovascular effects of anthracycline chemotherapy can be reduced or even prevented by the administration of RAAS inhibitors (Kalam and Marwick, 2013), then there must be a mechanism specific to this pathway which interacts with or links to the toxicological mechanism of anthracyclines. Activation of the RAAS promotes a range of cardiovascular effects, including the essential regulation of blood pressure and volume homeostasis. Angiotensin II release in response to physiological stimuli potentiates these effects through binding of AT1R on the cell surface. AT1R therefore has a direct effect upon myocardial structure and contractility because, in addition to blood pressure optimisation, angiotensin II strongly influences cardiac function by facilitating myocardial remodelling and inducing cardiomyocyte adaptive hypertrophy (Kurdi and Booz, 2011). Initially, these responses have a positive influence upon cardiovascular homeostasis and provide myocardial protection against acute physiological stressors. However, excess cardiac angiotensin II causes inflammation, oxidative stress and apoptosis, with prolonged stimulation resulting in cardiac hypertrophy and subsequent fibrosis, all of which have deleterious effects upon myocardial structure and function (Xu et al., 2010). As angiotensin II principally acts via AT1R, AT1R overexpression also induces cardiac hypertrophy and is associated with increased cardiovascular-related morbidity (Paradis et al., 2000). These cardiac effects are confirmed with clinical studies demonstrating that pharmacological blockade of AT1R prevents RAAS-induced hypertension and improves cardiovascular outcomes (Tadevosyan et al., 2011).

Although circulating systemic angiotensin II is the major source of this molecule within the adult cardiovascular system, a low level of RAAS stimulation has also been demonstrated locally within cardiomyocytes, fibroblasts and vascular smooth muscle tissue, resulting in low

basal levels in cardiac tissue (Kumar and Boim, 2009). This local angiotensin II synthesis appears to have tissue-specific functions including cell proliferation, growth and hypertrophic effects. Moreover, upregulation of intracellular RAAS and increased angiotensin II formation has been demonstrated in cardiovascular diseases such as heart failure, hypertension and myocardial infarction (Re, 2004). Taken together, several parallels between RAAS and the effects of anthracyclines upon the heart are evident, such as the induction of oxidative stress and formation of reactive oxygen species, causing ventricular cardiomyocyte apoptosis and necrosis. Widespread myocardial inflammation and fibrosis are observed in response to the myocardial damage, common to both RAAS dysregulation and anthracycline-mediated effects, with adverse myocardial remodelling culminating in harmful and unsustainable cardiovascular haemodynamics. These observations suggest a direct relationship between anthracycline-induced cardiotoxicity and the angiotensin signalling pathway.

The beneficial and detrimental effects of systemic angiotensin II in the heart are potentiated by AT1R binding and activation, with direct effects of angiotensin II upon cardiomyocytes including growth, apoptosis, remodelling and fibrosis (De Mello and Danser, 2000, Xu et al., 2010, Gao et al., 2020). The potential for local production of angiotensin II in the heart presents several opportunities to explain the cardiotoxic effects of anthracyclines. Human AC10 ventricular cardiomyocytes in this study were shown to produce and secrete angiotensin II during *in vitro* culture, but doxorubicin exposure did not appear to modulate these levels (**Table 4.4**). This implies that modulation of angiotensin II levels in cardiomyocytes is unlikely to be a major mechanism of anthracycline-induced cardiotoxicity, although it is still possible that the scale of such changes may necessitate larger cell volume or multicellular environments. Findings from Zheng et al. also favour the influence of systemic rather than local angiotensin II interaction; with elevated angiotensin II concentrations found within the paraventricular nucleus of the hypothalamus (Zheng et al., 2012), an area of the brain mediating cardiovascular effects via the baroreflex (Rostami et al., 2023).

A further putative mechanism is through the interaction or regulation of AT1R on cardiomyocytes either directly or indirectly by anthracyclines, with this receptor being the conduit between angiotensin II in the surrounding environment and cellular adaptive responses to this molecule. In this study, *in vitro* evaluation of the human AC10-CM cell line demonstrated both concentration and time-dependent increases in AT1R gene expression

following exposure of cells of doxorubicin. Evidence of AT1R gene expression activation by doxorubicin in ventricular cardiomyocytes is best determined between 8 and 24 hours of doxorubicin exposure, which aligns with the clinical half-life parameters of this drug. Peak gene expression measurements were identified at doxorubicin doses of 100nM and 250nM, with up to a 13-fold increase recorded relative to control. Conversely, 50nM doxorubicin demonstrated the lowest AT1R gene expression levels, and although doxorubicin doses of 50nM initiated small upregulations in gene expression, doses equal to and below 50nM are insufficient to activate all the AT1R receptors and translate into significant elevations in AT1R expression. Slight increases in AT1R gene expression relative to control were observed at 500nM doses, however, expression levels may be limited by the cytotoxic effects of doxorubicin on the cardiomyocytes. A disparity addressed in relation to determination of AT1R gene expression in this study was the appropriateness of GAPDH as a 'housekeeping' standardisation control for gene expression analyses, utilised widely in such methodologies (Backlund et al., 2009). Previous studies had indicated GAPDH to be a translational suppressor of AT1R expression, thereby negating its utility as a reference control in this specific context (Backlund et al., 2009). To circumvent this issue, the 'housekeeping gene' RPL13A was qualified to be a more appropriate comparator for calculation of gene expression in studies of the RAAS system.

In accordance with gene expression studies, cardiomyocytes were evaluated for AT1R protein expression following doxorubicin. At face value, although doxorubicin increased both gene and protein expression of AT1R, no direct relationship or agreement was observed between either concentration or exposure time effects with gene versus protein expression. Although increases of up to 2.7-fold protein expression were observed after 24 hours doxorubicin exposure, they did not increase beyond 48 hours and were not statistically significant. These results show a disparity in the relationship between angiotensin gene and protein expressions. One explanation for the weak correlation between expression of AT1R gene and protein levels could be a reduction in translation efficiency. Although 96-hour experimental time points were applied, a lag from increases in gene expression to induce protein expression may be responsible. Clinically relevant dosing schedules of doxorubicin has been shown to produce both immediate and late effects upon cardiac gene expression as determined through mRNA quantification (Boucek et al., 1999). The paired comparison time points utilised for the

protein expression studies may therefore have been too short to measure any significant in protein expression, with translation of proteins following a slower time course than AT1R gene transcription. In the presence of doxorubicin, changes in protein expression caused by anthracycline DNA intercalation and topoisomerase II inhibition, are known to cause persistent and progressive changes in cardiac gene expression (Boucek et al., 1999, Stamm et al., 2021). Therefore, higher cumulative doses of doxorubicin may be required to produce detectable alterations in gene and protein expression.

Protein stability in the presence of doxorubicin may also influence AT1R expression levels, with evidence of reduced AT1R mRNA concentrations and receptor density in damaged ventricular myocardium compared to healthy myocardium (Haywood et al., 1997). The resulting increases in free angiotensin II could have a negative feedback effect upon AT1R expression, with angiotensin II previously observed to decrease AT1R mRNA expression and promote inflammation. Furthermore, reactive oxygen species and free radial formation have also been shown to have post-transcriptional and translational effects on AT1R receptor expression, mediated through increases in angiotensin II concentrations (Nickenig et al., 2000). A further explanation could be modulated stability of the post-transcriptional and post-translational AT1R gene or protein, as has been reported in the case of insulin and AT1R (Nickenig et al., 1998, Paukku et al., 2012). Consistent with observations of doxorubicin, insulin also induces a time- and concentration-dependent upregulation of AT1R gene expression, with maximal effect observed at 24 hrs (Nickenig et al., 1998). However, in this context, upregulation was shown to be due to stabilisation of AT1R mRNA and a subsequent doubling of its stability, rather than exclusively de-novo gene transcription (Nickenig et al., 1998). It remains to be determined whether a comparative mechanism could also be associated with doxorubicin-induced upregulation of AT1R, with the caveat that the disparity could be more aligned to AT1R protein expression, and thus potentially post-translational rather than transcriptional effects (Nickenig et al., 2000).

Despite the fact that doxorubicin-induced AT1R protein expression was not shown to linearly related to concentration and exposure related effects, analysis of the in-situ cellular expression of AT1R showed this to be increased in response to clinically relevant exposures to doxorubicin, as determined by immunofluorescence staining of AC10-CMs. Crucially, AT1R overexpression has been shown to increased left ventricular hypertrophy and adverse cardiac

remodelling more effectively than elevations in angiotensin II levels, therefore indicating that receptor expression and density could be the regulatory force driving these cardiovascular adaptations (Reudelhuber et al., 2007). When exposed to anthracyclines, increased AT1R expression in ventricular cardiomyocytes would therefore intensify the adverse cardiovascular effects of angiotensin II, including cardiovascular structural remodelling, interstitial fibrosis, and subsequent cardiac dysfunction. This mechanism draws strong parallels to those observed in congestive cardiac failure and myocardial ischaemia, both of which produce RAAS upregulation and increased angiotensin II expression in response to cardiomyocyte damage (Xu et al., 2007). Taken together, in conjunction with the previously reported inhibition of doxorubicin-mediated cellular hypertrophy by exposure to ARBs, these observations strongly support the mechanistic hypothesis that a relationship exists between anthracycline-induced cardiotoxicity and modulation of the angiotensin signalling pathway in cardiomyocytes (Rockley, 2018). *In vivo* research studies support this hypothesis with anthracycline-induced cardiotoxicity histological changes such as cytoplasmic vacuolisation and myofilament loss in cardiomyocytes being attenuated by knocking-out AT1R in mice exposed to acute and chronic doxorubicin treatment schedules. By preventing angiotensin II activity and signalling, adverse changes in left ventricular morphology and reductions in cardiac ejection fraction were also prevented (Toko et al., 2002). In the context of anthracycline-induced cardiotoxicity, specific angiotensin II inhibition could therefore crucially prevent development and progression of anthracycline-induced cardiac failure, offering an explanation for clinical studies to date (Cardinale et al., 2015).

Extracellular angiotensin II, released principally from the lungs and kidneys, couples to AT1R located on cardiac cell membranes to initiate cellular proliferation, hypertrophy, and increase cardiac contractility. Importantly, the RAAS also exhibits potent intracellular action, with angiotensin II capable of influencing transmembrane electrolyte currents and myocardial contractility (Forrester et al., 2018). The results of the immunofluorescence experiments demonstrated a greater intensity of AT1R expression centrally within the cardiomyocytes compared to the cell periphery. As the methodology did not include a specific cell membrane permeabilisation step permissive of antibody cell entry, this localisation is unlikely to be intracellular and play a role in cardiac homeostasis, as previously reported (Tadevosyan et al., 2010, Qi et al., 2021). A more likely explanation is the congregation of receptors on the apical

cell surface, as opposed to the cell-cell interface, of a cellular monolayer. However, to conclusively establish this, further cell fractionation analyses are warranted.

With regards to the clinical situation, angiotensin II is the driving force for the physiological and pathological effects of the RAAS system upon the cardiovascular system, with a key role in the development and progression of cardiac failure. Studies measuring plasma and cardiac angiotensin II have demonstrated that in patients with hypertension and cardiac failure, baseline angiotensin II concentrations are significantly higher than control populations (Catt et al., 1969, Serneri et al., 2001, Cui et al., 2019). Consequently, inhibition of angiotensin II production, mediated through ACE inhibition, or blocking of AT1R and thus downstream signalling from angiotensin II are recognised systemic therapeutic approaches for hypertension and cardiac failure, supported by strong evidence from large-scale cardiovascular research trials (CONSENSUS, 1987, SOLVD, 1991, Jong et al., 2003).

The dynamic relationship and balance between angiotensin II levels and AT1R expression and activity is crucial for regulation of normal cardiac physiology. Thus, any factors which disturb, dysregulate or imbalance this relationship would have potentially supra-physiological and detrimental cardiac effects, such as hypertension, cardiac dysfunction and failure. In this context, despite the clear therapeutic efficacy of ACEi and high degrees of success in the clinic, subsequent research measuring RAAS pathway activity in patients with heart failure has revealed persistence of elevated angiotensin II concentrations even with ACEi therapy. These findings are clinically significant given the adverse cardiovascular consequences of long-term angiotensin II exposure, as evidenced in patients with heart failure, where sustained increases in angiotensin II are associated with worsening heart failure and higher mortality (Roig et al., 2000, van de Wal et al., 2006). This phenomenon is described within the literature as an angiotensin II and aldosterone 'escape' mechanism. During the early initiation phases of ACEi therapy, angiotensin II concentrations fall, due to blockade of RAAS pathway mediated through the ACE, lowering angiotensin II production. However, in response to low angiotensin II production, renin activity increases (due to the absence of negative feedback) and drives angiotensin I synthesis. As angiotensin II is produced principally from angiotensin I conversion by ACE, this ACE-dependent pathway 'overproduces' angiotensin II and ACE inhibition becomes overwhelmed (Roig et al., 2000, Fildes et al., 2005, van de Wal et al., 2006). Such an activity implies a 'threshold' of angiotensin II levels, above which angiotensin initiates adverse

morphological effects upon the heart. With regards to anthracycline therapy, it is conceivable that the initial loss of cardiomyocytes lowers this threshold and/or modulates activity levels of the AT1R mediated activity relationship of angiotensin II. Interindividual variabilities in this threshold in cancer patients may therefore provide an explanation for the subsequent development of cardiotoxicity following anthracycline therapy. One such mechanism is the presence of polymorphic variants in ACE within the patient population, with those exhibiting high activity ACE producing higher concentrations of angiotensin II, and thus exceeding the toxicity threshold more rapidly than those with genotypes associated with lower ACE activity. Alternatively, patients at higher risk of anthracycline-induced cardiotoxicity may exhibit higher systemic levels of angiotensin II, which when coupled with anthracycline-induced upregulation of AT1R within cardiac tissue results in higher activity of angiotensin II signalling pathways and consequent cellular responses in the heart.

In order to evaluate relationships between angiotensin II levels, pharmacogenetic variation in ACE, and development of anthracycline-induced cardiotoxicity and subsequent heart failure, it was important to establish a robust quantitative methodology for determination of human plasma angiotensin II levels. Within the literature, there is limited evidence regarding the influence of plasma sample storage upon measured angiotensin II concentrations. Previous studies have indicated that samples could be stored at either minus 4°C or minus 20°C for at least 4 weeks without altering measurements of plasma ACE activity, and reliably reproducible for up to three freeze-thaw cycles (Jalil et al., 1999, Gao et al., 2018). In the current study, statistically significant decreases in angiotensin II levels were observed across freeze thaw cycles, an effect observed across all the samples (**Figure 4.16**). These results therefore emphasise the importance of sample handling when measuring RAAS pathway derivatives and recommend early analysis of the samples. The mean angiotensin II concentration found in the healthy volunteers in this study (32.3 ± 16.8 pg/mL) was consistent with direct angiotensin II measurements obtained from validation studies, in which normotensive subjects displayed angiotensin II values between 5 to 55 pg/mL (Catt et al., 1969, Chappell, 2016).

In terms of the cancer patients, all of which were female and from a breast cancer cohort, the mean concentration of plasma angiotensin II was found to be much higher than in the healthy volunteer study participants and the published validation studies, with a mean value of 57.1 ± 40.4 pg/mL. This is especially noteworthy since females have reportedly lower plasma

angiotensin II levels than males, as indicated in **Figure 4.13** with values of 22.4 ± 8.7 pg/mL and 58.5 ± 34.2 pg/mL for females and males, respectively. The potential reasons for this are an increase in mean age of the breast cancer cohort (58.8 ± 8.7 years) compared to the mean of the female healthy volunteers (40.8 ± 8.6 years) (p=0.002), the presence of cancer, ethnicity, and cardiovascular co-morbidities which are known to increase angiotensin II levels (Chappell, 2016).

No statistically significant difference between the mean baseline plasma angiotensin II concentrations of cardiotoxicity and non-cardiotoxicity patients was observed within this study (59.4± 33.0 vs. 89.4± 56.6 pg/mL, p=0.245). This implies that baseline plasma angiotensin II levels are not a predictor for the development of anthracycline-induced cardiotoxicity, with amongst the highest angiotensin II concentrations found in the non-cardiotoxicity group. Therefore, the concept of an elevated baseline threshold of angiotensin II levels being responsible for the development of the cardiotoxicity phenotype appears not to be the case. These findings do however strengthen the gene expression analysis results, which indicated AT1R upregulation in the presence of doxorubicin to regulate subsequent cardiotoxicity responses. Another reason for this observation could be that no significant differences existed between the groups when the samples were obtained at baseline, with discernible differences in angiotensin II levels developing during and after chemotherapy treatment. Angiotensin II levels would then increase in response to cardiovascular physiological stress caused by anthracycline chemotherapy, with the patients displaying the highest peak angiotensin II levels developing cardiotoxicity. This hypothesis has been explored within animal models, with Zheng et al. (2012) demonstrating a three-fold increase in plasma angiotensin II in response to doxorubicin exposure (Zheng et al., 2012). Furthermore, angiotensin II levels may not change during chemotherapy, but the doxorubicin-induced increased AT1R expression and modulation may drive the cardiovascular manifestations of angiotensin II, culminating in anthracycline-induced cardiotoxicity.

Whilst no statistically significant differences were observed in ACE genotype between the cardiotoxicity and no cardiotoxicity patients, in contrast to other cardiomyopathies, these results advance our understanding of anthracycline-induced cardiotoxicity and angiotensin signalling. Anthracycline-induced cardiotoxicity is a multi-factorial process, with RAAS activation based also upon physiological stress, the severity of myocardial injury and genetic

susceptibility. It is the genetic susceptibility which is of particular interest and has led to evaluating the significance of the ACE genotype on development of the cardiotoxicity phenotype. In the present study, genetic influences upon angiotensin II levels and whether an association exists between the ACE genotype and the cardiotoxicity phenotype was evaluated. Genotyping of the anthracycline pharmacogenomic patient cohort displayed a broader spread of genotypes, including I/I genotype patients. Within this cohort, patients were grouped based upon cardiotoxicity status and analysed according to their ACE genotype. The distribution of ACE genotype did not demonstrate a significant difference between individual genotype, although cardiotoxicity patients displayed a propensity towards the deletion allele (92.9% classified as either D/D and I/D genotypes), whilst patients without cardiotoxicity demonstrated a lower distribution of combined D/D and I/D genotypes (85.7%). This distribution towards the deletion allele is even more pronounced when compared to other studies of control populations demonstrating differing frequencies of the D/D, I/D and I/I genotypes (0.31, 0.20, 0.49) (Lindpaintner et al., 1995).

The deletion allele has previous been associated with cardiovascular disease (e.g. myocardial infarction, hypertension) and cardiac remodelling (Chen et al., 2013, Yuan et al., 2017), and may therefore demonstrate a stronger association with cardiotoxicity in large cohort studies. Patients with the I/I genotype produced the highest mean concentrations in angiotensin II genotypes (mean ± s.d., 82.9 ± 2.3 pg/mL) compared to I/D and D/D genotyped patients (59.8 ± 44.8 pg/mL and 56.6 ± 39.2 pg/mL, respectively). These results are surprising given previous research demonstrating serum ACE levels are highest in subjects with ACE genotype D/D, and lowest in I/I genotyped subjects (Biller et al., 2006). Serum ACE levels were not measured during this study, however, in vivo research has demonstrated that serum ACE levels increase in response to chronic doxorubicin treatment (Okumura et al., 2002). Co-administration of ACEi in these studies not only prevented this rise in serum ACE concentrations, but also prevented cardiac dysfunction and improved overall mortality (Okumura et al., 2002, Sacco et al., 2009). Patients with hypertension, ischaemic heart disease and heart failure have been successfully evaluated for the ACE genotype, however, this study is the first to evaluate the association between angiotensin II concentrations and ACE genotypes in patients with anthracycline-induced cardiotoxicity.

A lack of variation in serum angiotensin II concentrations was observed within the patients identified as having the I/I ACE genotype (**Figure 4.24**), which could be accounted for by the small sample size within this genotype-specific group. The previous studies evaluating both the ACE genotype and serum ACE levels found that not only did the I/I genotype display the lowest serum ACE concentrations, but these patients also demonstrated the lowest variation in ACE levels. Within healthy individuals, stable concentrations of serum ACE are recorded, however, larger inter-individual variability is frequently observed within cohorts, with genotype-specific reference values aiding their interpretation patients (Rigat et al., 1990, Biller et al., 2006). Conditions strongly linked to the immune response such as sarcoidosis, a multi-systemic inflammatory disease causing widespread granuloma, produce marked elevations in patients' serum ACE measurements, yet interestingly, serum ACE levels still remain lower in patients with the I/I genotype, and again displaying limited variability with respect to the other ACE genotypes (I/D, D/D) (Furuya et al., 1996, Sharma et al., 1997). Given that ACE activation is required to convert angiotensin I to angiotensin II, restrictions in circulating serum ACE in patients as seen with the I/I genotype, would be expected to produce little change in the mean angiotensin II concentrations. From the ACE genotype, angiotensin II and cardiotoxicity phenotype studies, the precise implications of these observations are yet to be determined. Potential hypotheses to explore in future include; whether the I/I genotype offers a protective effect for the development of cardiotoxicity, whether indeed these patients display a milder cardiotoxicity phenotype, and with respect to ACE inhibitor therapy, whether patients with the I/I genotype exhibit a better response to ACE inhibitors for the treatment and prevention of cardiovascular toxicity. These proposed studies may be limited by the overall lower prevalence of the I/I genotype within the general population, relative to the other ACE genotypes (I/D, D/D). Therefore, recruiting a larger patient cohort to encompass requisite numbers of the I/I specific genotype, and reassessing its relationship to the cardiotoxicity phenotype would be clinically valuable.

There remains a lack of data in humans evaluating serum ACE or other clinical biomarkers of RAAS activity in response to anthracycline chemotherapy, these studies provide additional stimulus to evaluate whether these components of the RAAS pathway can predict cardiotoxicity development. Although the study was unable to identify patients with anthracycline-induced cardiotoxicity based upon ACE genotype, a larger study evaluating the

ACE genotyping in patients receiving anthracycline chemotherapy would support and help to establish the significance of the deletion genotype within cardiotoxicity patients.

4.7.1 Conclusions

Despite clinical evidence supporting the role of ACEi for the treatment and prevention of anthracycline-induced cardiotoxicity, little is known regarding the mechanism within this specific setting. Whilst haemodynamic changes and reductions in myocardial wall stress may play a role, the direct relationship between the angiotensin signalling pathway and cardiotoxicity have yet to be determined. This chapter recognises the importance of AT1R, as increases in AT1R gene expression within cardiomyocytes exposed to doxorubicin were detected. Although this was not shown to translate immediately into significant increases in AT1R protein expression, these results reinforce the role of the angiotensin signalling pathway in cardiotoxicity. Further evidence of RAAS activation was evaluated, with plasma angiotensin II concentrations measured and ACE genotyping determined in patients diagnosed with breast cancer who had received anthracycline-based chemotherapy. Long-term follow-up data established the cardiotoxicity statuses of these patients and indicates that cardiotoxicity may develop many years post-anthracycline chemotherapy, with this study representing one of the longest follow-up cohorts within cardio-oncology. Plasma angiotensin II concentrations pre-chemotherapy were found not to be predictive of anthracycline-induced cardiotoxicity development, whilst the deletion ACE genotype may indicate propensity towards cardiotoxicity. Further research studies evaluating other aspects of the RAAS pathway are required, with these analyses providing the stimulus for identifying strategies of cardiotoxicity mitigation using ACEi and ARBs.

Given the restrictions imposed by the COVID-19 pandemic, sufficient time to optimise hiPSC-CM modelling protocols and conduct experiments was not available, therefore acknowledging this limitation within the study. Without these mandatory time constraints upon our research, experiments completed using the hiPSC-CMs would have produced valuable data to analyse the effects of anthracyclines upon *in vitro* cardiomyocytes, supporting our hypotheses regarding the potential cardiotoxicity and cardioprotective mechanisms.

Chapter 5. Anthracycline-induced cardiotoxicity risk factors and monitoring

5.1 Introduction

Chronic anthracycline-induced cardiotoxicity usually presents in the clinic as heart failure, with patients identified as having severe cardiotoxicity (cardiomyopathy defined by LVEF <40% or symptomatic cardiac failure) demonstrating a ten-fold increase in all-cause mortality compared to mild, moderate or no evidence of chemotherapy-induced myocardial injury (López-Sendón et al., 2020). Furthermore, comparisons of survival outcomes associated with different heart failure aetiologies have shown doxorubicin-induced cardiomyopathy to have one of the poorest survival rates (hazard ratio 3.46), with a two-year mortality greater than 50%, and associated with a higher long-term risk of heart transplantation or left ventricular assist device (LVAD) implantation (Felker et al., 2000, Nadruz et al., 2019).

Prior to the introduction of cardiovascular imaging techniques to identify cardiotoxicity, the early anthracycline-induced cardiotoxicity research studies relied solely upon the detection of congestive cardiac failure symptoms (von Hoff et al., 1979). Given the poor prognosis of congestive cardiac failure, delays in recognising cardiac dysfunction and initiating treatment considerably increases patient mortality (McDonagh et al., 1997, Davies et al., 2001). Therefore, diagnosing anthracycline-induced cardiotoxicity in patients with cancer at the earliest possible stage is imperative to reducing morbidity and mortality in an already high-risk patient population.

Although earlier diagnosis and treatment of subclinical myocardial dysfunction improves clinical outcomes, measurement of LVEF, by echocardiography or multi-gated acquisition (MUGA) scan, may no longer be the most reliable predictor of future congestive cardiac failure (CCF). Analysis of three phase III studies identified the limitations of relying upon left ventricular function measures as a predictive strategy for future CCF (Feldmann et al., 1992, Swain et al., 1997a, Swain et al., 1997b). In addition to reporting a high cardiac event rate in the patients receiving doxorubicin chemotherapy and an overall CHF incidence of 5.1%, they observed 11 of the 32 patients (34%) to have a normal LVEF, until clinical presentation with CCF and subsequent reduction in LVEF were diagnosed (Swain et al., 2003). These unexpected results demonstrate that LVEF is perhaps not the anticipated high-sensitivity marker required

to accurately evaluate early, subclinical cardiotoxicity and influence treatment, with time to cardiac decompensation being much shorter than expected. A similar conclusion was reached by Doyle et al. (2005) who evaluated a large retrospective cohort of older breast cancer patients receiving anthracycline chemotherapy. Despite screening patients for pre-existing cardiac dysfunction and subsequent cardiotoxicity monitoring for one-year post chemotherapy, Doyle et al. observed that the cumulative incidence of anthracycline-induced cardiotoxicity had more than doubled after 5-years of follow-up (4% to 10%). Their analysis confirmed a high incidence of anthracycline-induced cardiomyopathy in patients with a previously normal LVEF (Doyle et al., 2005). Both sets of authors concluded that LVEF, regardless of whether assessed by echocardiography or MUGA, is an insufficient marker for detecting early cardiomyocyte damage, with more sensitive measures for cardiotoxicity risk prediction required for clinical decision-making, to prevent chemotherapy-induced morbidity and mortality.

Strategies for identifying subclinical anthracycline-induced cardiotoxicity (i.e. before the onset of heart failure symptoms) have therefore been applied within clinical practice to enhance cardiotoxicity detection and reduce the progression of cardiotoxicity. Using a combination of advanced cardiovascular imaging and cardiac biomarkers, patients within this subclinical cardiotoxicity phase can be identified and treated before clinical deterioration. Ewer et al. (1984) first demonstrated that despite having a normal ejection fraction on cardiac imaging, patients receiving anthracycline chemotherapy could in fact have advanced histological changes on cardiac biopsy, crucially recognising that subtle myocardial changes precede reductions in ejection fraction and onset of clinical cardiotoxicity (Ewer et al., 1984). With LVEF being a composite measure of longitudinal, circumferential and radial myofibril shortening, reductions in one dimension of cardiac function e.g. longitudinal, can be compensated for by other parameters to maintain ejection fraction despite myocardial damage. Therefore, whilst remaining a useful prognostic indicator, LVEF has been shown be an insensitive measure for detecting the subtle changes of myocardial injury and diagnosing early cardiotoxicity (Plana et al., 2014, Lang et al., 2015, Harkness et al., 2020).

Advanced echo technologies such as global longitudinal strain (GLS), tissue doppler imaging and speckle-tracking echocardiography have provided more sensitive measures of cardiac functional decline, whilst 3-dimensional (3-D) echo has superiority over 2-D echo, and strongly

correlates with cardiac MRI (CMR) for left ventricular assessment (Santoro et al., 2017). The introduction of CMR has also provided valuable insights into anthracycline-induced structural changes and helped further establish our understanding of subclinical cardiotoxicity. The enhanced sensitivity of CMR techniques has provided clinicians with the potential to recognise the early tissue changes associated with anthracycline exposure prior to the development of cardiac dysfunction. Whilst characterisation of the myocardium using extracellular volume, late gadolinium enhancement (LGE), T1 and T2 mapping techniques within CMR has provided deeper insight into the spectrum and progression of cardiotoxicity associated with anthracycline chemotherapy (Haslbauer et al., 2019, Seraphim et al., 2019). As the evidence has shown, a reduction in LVEF is considered a late change along the cardiotoxicity spectrum, and as the long-term treatment options are still limited for patients, earlier intervention is required to ensure full functional recovery and prevent progression towards symptomatic cardiac failure. Multi-modality imaging has therefore strengthened anthracycline-induced cardiotoxicity detection in patients with cancer, with early detection of ultra-structural myocardial changes able to accurately predict subsequent LVEF decline and influence treatment decisions before progression towards CCF.

The role of cardiac biomarkers in patients has been investigated for anthracycline-induced cardiotoxicity risk stratification. Recognising the capability of cardiac troponins to detect early phase myocardial injury, several studies have applied their high diagnostic and prognostic potential to evaluate patients for cardiac injury post-chemotherapy. Historically, it had only been possible to diagnose anthracycline-induced cardiotoxicity following overt functional impairment on cardiac imaging or the presence of clinical symptoms. However, Cardinale et al. reported the strong association between increased serum troponin-I and the later development of impaired left ventricular systolic dysfunction (Cardinale et al., 2000). After anthracycline chemotherapy, patients displaying persistently raised troponin-I levels were identified as high risk for development of cardiotoxicity, with 84% of patients experiencing a major cardiac event during the first year of follow-up. Those with elevated troponin-I were more likely to experience a significant decrease in LVEF within the first month post-anthracycline treatment, which worsened during the following months (6.8% LVEF reduction at one month to an 18.2% reduction at twelve months) (Cardinale et al., 2004). Meta-analysis data provides strong evidence for troponin risk stratification, with a persistently negative

troponin throughout anthracycline treatment indicative of low anthracycline-induced cardiotoxicity risk (negative predictive value 93%) (Michel et al., 2020a). The ability of troponin to detect early phase myocardial injury prior to signs of cardiac dysfunction conveys a significant advantage throughout cancer treatment, as the earliest stages of myocardial injury can be rapidly detected, whilst those at risk of late cardiotoxicity can be closely monitored and receive early pharmacological intervention (Horacek et al., 2014). Interestingly, meta-analysis data evaluating troponin levels in paediatric patients failed to establish an association between an elevated troponin and left ventricular dysfunction, whilst other paediatric studies revealed no significant increase in troponin at all (Kismet et al., 2004, Soker and Kervancioglu, 2005, Michel et al., 2020b). This provides additional evidence in support the hypothesis that anthracycline-induced cardiotoxicity in paediatric patients and childhood cancer survivors should be considered as a separate clinical entity from that seen in adults.

Natriuretic peptides (ANP, BNP and NT-proBNP) have also been studied and have shown promise as clinical biomarkers for anthracycline-induced cardiotoxicity. Clinically, elevated plasma concentration of these peptides can be used to support a diagnosis of heart failure and for establishing clinical prognosis. They are released in response to increased intra-cardiac pressures and volume, therefore elevated BNP levels are used to diagnose underlying cardiac impairment (Cowie et al., 1997, McDonagh et al., 2021). Although plasma natriuretic peptide increases are associated with the administration of anthracycline chemotherapy, in adults the overall correlation with LVEF decreases remains inconsistent, especially when compared to the meta-analysis data from cardiac troponins. Serial measurements of BNP during and after anthracycline chemotherapy could enhance early detection of anthracycline-induced cardiotoxicity, however, prospective studies with larger patient cohorts incorporating cardiac risk factors are required to establish natriuretic peptides as a clinically useful biomarker for cardiotoxicity (Horacek et al., 2014, Michel et al., 2020a). The evaluation of natriuretic peptides in childhood cancer again provides an interesting contrast to adult patients, with BNP/NT-proBNP increases reliably detected in response to anthracycline chemotherapy and associated with acute anthracycline-induced cardiotoxicity. Whilst further evidence is required to support the use of BNP/NT-proBNP as a standalone biomarker and for screening of late-onset cardiotoxicity in childhood cancer survivors, it again suggests that a

patient's stage of cardiac development and maturation could determine the most appropriate screening strategy for anthracycline-induced cardiotoxicity detection (Michel et al., 2020b).

Advanced assessment and screening of cardiotoxicity has represented a major breakthrough since the early days of histological assessment and manifestation of clinical syndromes. The emergence of blood biomarkers as a strategy for the early detection of cardiotoxicity represents another significant innovation within cardio-oncology. A symbiotic relationship should exist between blood biomarkers and cardiac imaging, with both having the ability to detect cardiotoxicity well before the manifestation of clinical syndromes and positively influence clinical outcomes. Blood biomarkers have provided important insight into the expectant changes within the myocardium which could be detected by cardiac imaging, whilst significant imaging findings identify other potential biomarkers that can be screened for in patients treated with chemotherapy (Ky et al., 2014, Tan and Lyon, 2018). A clinical biomarker strategy, based upon cardiac imaging and known histological changes during the course of anthracycline-induced cardiotoxicity, has the strong potential to enhance anthracycline-induced cardiotoxicity detection (**Table 5.1**). The future of cardio-oncology will almost certainly integrate the combination of imaging and circulatory biomarkers, to detect cardiotoxicity at the earliest stage and to promote long-term cancer survival.

Table 5.1 Potential strategy for clinical biomarker cardiotoxicity surveillance in patients with cancer treated with anthracycline chemotherapy

Early → Late			
Oxidative stress and inflammation	Myocardial necrosis, cardiomyocyte injury	Neurohormonal	Fibrosis

5.1.1 Risk factors associated with anthracycline-induced cardiotoxicity

Given the substantial impact of chemotherapy-induced cardiotoxicity and the negative influence upon cancer patient survival, early detection is imperative to maximising patient quality of life and continuing to improve long-term cancer outcomes. Several risk factors have been proposed within the literature for predicting the development of anthracycline-induced cardiotoxicity and informing surveillance decisions. The therapy-related risk factors so far identified are cumulative anthracycline dose, shorter duration of intravenous infusion,

previous anthracycline administration, mediastinal radiotherapy, and concurrent chemotherapy (e.g. trasutzumab, cyclophosphamide). Patient-related risk factors include age, pre-existing cardiac disease, hypertension and female gender (von Hoff et al., 1979, Scully and Lipshultz, 2007, Gavila et al., 2017, Smith et al., 2010).

Through extrapolation of these known factors, prediction models estimating the probability of developing congestive cardiac failure, ischaemic heart disease and stroke have been derived (Armenian et al., 2018). By combining genetic analysis with clinical factors, Armenian et al. established a risk prediction model which correctly identified 75% of patients in the high-risk group who went on to develop anthracycline cardiotoxicity, whereas in the low-risk group 96% of patients did not develop cardiotoxicity (Armenian et al., 2018). These models are validated in survivors of childhood cancers, but accurate risk prediction for the older adult population is still being explored in order to refine cardiomyopathy surveillance and reduce anthracycline-related morbidity (Chow et al., 2015).

5.1.2 Cardiovascular risk assessment in patients with cancer

After cancer, cardiovascular disease is the second most common cause of death in developed countries. It is a disease seen most frequently in the ageing population reflecting the demographic of the majority of cancer patients. Whilst improvements in cancer diagnosis and therapy continue to establish a good quality of life for many cancer patients, these patients are susceptible to prolonged cardiovascular risk factor exposure, resulting in increased cardiovascular mortality in the older cancer patient population. Furthermore, within the last 5 years the introduction of targeted therapy and immunotherapy for many common cancers has transformed the prognosis for some patients, who may now have a reasonable expectation of long-term survival.

Cardiovascular risk has been demonstrated to be up to 5 times higher in cancer survivors than in the general population, with the risk of cardiovascular morbidity shown to be higher than tumour recurrence within cancer survivor populations even decades after diagnosis (Oeffinger et al., 2006, Schindler et al., 2016). In a recent Lancet publication, Strongman *et al*. (2019) observed an increased risk of heart failure in survivors of 10 out of 20 site-specific cancers compared with that of controls. Survivors of haematological, oesophageal, lung, kidney and ovarian cancers were at least 50% more likely to experience cardiomyopathy or

heart failure than people with no prior cancer diagnosis (Strongman et al., 2019). Similarly, elevated risks of arrhythmia, pericarditis, coronary artery disease, stroke, and valvular heart disease were also recorded for multiple cancers, including haematological malignancies (Oeffinger et al., 2006, Strongman et al., 2019). The elevation in cardiovascular risk can be attributed to the shared risk factors with cancer (e.g. smoking, obesity, ageing, alcohol and diabetes mellitus) combined with the cardiotoxic effects of chemotherapy and radiotherapy (Koene et al., 2016).

A high baseline cardiovascular risk significantly increases the risk of cardiotoxicity, especially in older patients, and screening to facilitate modification of these risk factors prior to initiation of therapy is recommended to improve cardiovascular outcomes. Patients' risk can be quantified using an evidence-based score (HeartScore, QRISK-3 or 2013 ACC/AHA guideline on the assessment of cardiovascular risk), and then those at increased risk can be offered appropriate preventative lifestyle advice and treatments (Rabar et al., 2014, Stone et al., 2014, Piepoli et al., 2016). However, despite their increased cardiovascular risk, cancer survivors have been shown to be less likely to receive primary cardiac prevention therapies than the general population, further enhancing their risk of future cardiovascular events (Armenian et al., 2016b, Matthews et al., 2016, Chidwick et al., 2018, Hope et al., 2019).

5.1.3 Hypertension and anthracycline-induced cardiotoxicity

Hypertension is recognised as one of the strongest modifiable risk factors for future cardiovascular morbidity and mortality, with elevated blood pressure recognised as the leading global contributor to premature death in 2015 (Global Burden of Metabolic Risk Factors for Chronic Diseases, 2014, Forouzanfar et al., 2017). European clinical guidelines define hypertension as a systolic blood pressure (SBP) ≥140 mmHg, and/or a diastolic blood pressure (DBP) ≥90 mmHg (Williams et al., 2018). Strong evidence exists that lower diagnostic thresholds (i.e. systolic: 120-139 mmHg, diastolic: 80-89 mmHg) are also associated with high rates of cardiovascular-related morbidity and mortality (Whelton et al., 2018).

Cancer registries report over a third of cancer patients with a concurrent diagnosis of hypertension, with meta-analysis data providing evidence for an association between multiple cancer types and hypertension (Piccirillo et al., 2004, Cohen et al., 2019, Seretis et al., 2019). Compared with the general population, the incidence of hypertension is far greater in cancer

survivors. This has strong implications for long-term cancer survival as hypertension is associated with coronary artery disease, heart failure, valvular heart disease and cardiac arrhythmia-related mortality (Armstrong et al., 2013). Cancer and hypertension share several risk factors including smoking, obesity, and diabetes. However, several cancer therapies, specifically anti-VEGF therapy and tyrosine kinase inhibitors, are associated with the development of hypertension. Radiotherapy is another recognised cardiovascular risk factor, with mediastinal radiation of the coronary arteries and myocardium resulting in an increased risk of coronary artery stenoses and myocardial fibrosis (Spetz et al., 2018), whilst head and neck radiotherapy is a recognised mediator of hypertension and hypertensive crises through baroreflex failure (Sharabi et al., 2003).

Since the earliest cardiotoxicity studies, arterial hypertension has been recognised as a significant risk factor for anthracycline-induced cardiotoxicity (von Hoff et al., 1979). Subsequent clinical studies have supported this relationship, with pre-existing hypertension associated with higher rates of left ventricular systolic dysfunction in patients treated with anthracyclines for lymphoma and breast cancer (Pinder et al., 2007, Hershman et al., 2008, Szmit et al., 2014). The haemodynamic stress and subsequent cardiovascular damage induced by poorly controlled hypertension exacerbates the cardiomyocyte injury produced by anthracycline exposure, as evidenced by increased rates of both subclinical cardiotoxicity and congestive cardiac failure (Hequet et al., 2004, Drazner, 2011). These findings have significant adverse implications for patients, as demonstrated by Szmit et al. (2014), who found that the presence of pre-existing arterial hypertension in patients receiving anthracycline chemotherapy led to more treatment cycle delays, doxorubicin dose reductions and premature treatment discontinuations (Szmit et al., 2014).

Although arterial hypertension is a strong predictor for anthracycline-induced cardiotoxicity, the effects of anthracycline chemotherapy upon blood pressure and developing subsequent hypertension have not yet been fully appraised. There is increasing evidence on a cellular and hormonal level to support this hypothesis, with oxidative stress mechanisms common to both anthracycline-induced cardiotoxicity and myocardial damage caused by hypertension (Rodrigo et al., 2011, Carrasco et al., 2021). RAAS over-stimulation, specifically elevated angiotensin II, is considered responsible for cardiovascular inflammation and progressive cardiac fibrosis in patients with clinical hypertension (Hunter and Chien, 1999). This provides

a crucial link to anthracycline-induced cardiotoxicity, with these described histopathological changes also described in patients treated with anthracycline chemotherapy (Billingham et al., 1978). Through abnormal cell signalling and the synthesis of cytotoxic molecules (i.e. reactive oxygen species), the strong possibility of interdependent relationship between anthracycline-induced cardiotoxicity and hypertension is recognised (Seddon et al., 2007, Kuriakose et al., 2016). This is further supported by the use of RAAS inhibitors, which are administered in patients for prevention and treatment of cardiovascular wall stress and myocyte damage induced by both pathologies (Cardinale et al., 2006).

Recent cardio-oncology clinical guidelines strongly advocate the screening of arterial hypertension in patients prior to receiving anthracycline chemotherapy, initiating anti-hypertensive treatment when indicated (Lyon et al., 2022). Whilst these international guidelines propose monitoring and treatment of cardiovascular risk factors (i.e. hypertension, diabetes, hypercholesterolaemia) beyond the completion of chemotherapy, there remains a paucity of clinical data detailing the incidence of hypertension following anthracycline chemotherapy. The clinical implications of the ACE/angiotensin II/AT1R axis activation should therefore be explored, through analysis of changes in blood pressure and the relationship to anthracycline-induced cardiotoxicity.

5.1.4 Anthracycline-induced cardiotoxicity monitoring

A major clinical issue with the development and management of anthracycline-induced cardiotoxicity is efficient monitoring of susceptible individuals and the timescale of its development. Following administration, patients may remain asymptomatic for several years and often only present at the clinic only when significant symptoms appear. This is further compounded by a lack of correlation between systemic biomarkers of cardiac dysfunction and myocardial damage, with the development of anthracycline-induced heart failure. Identifications of these correlations and appropriate utilisation of imaging modalities would permit optimisation of clinical responses to treatment and the implementation of long-term cardio-oncology surveillance.

Several international cardio-oncology guidelines have been published to support clinical decision making during and after chemotherapy. The consensus appears to be that cardiovascular imaging surveillance should be determined by patient risk stratification, taking

into consideration the baseline cardiovascular risk and anthracycline dose (Čelutkienė et al., 2020, Curigliano et al., 2020, Stone et al., 2021). Although early post-chemotherapy strategies for anthracycline-induced cardiotoxicity monitoring are well-defined (i.e. 12 months following the final cycle of chemotherapy), the challenges surrounding long-term cardiotoxicity surveillance understandably creates a lack of clarity and uniformity within the guidelines. Perhaps due to uncertainty regarding the pathophysiology and long-term clinical course of anthracycline-induced cardiotoxicity within the cardio-oncology community, clinical surveillance intervals vary between guidelines. International recommendations currently advice interval scanning anywhere from 1 to 5 years, with the follow-up duration post-chemotherapy unspecified.

5.1.4.1 Anthracycline-induced cardiotoxicity monitoring in paediatric cancer survivors

In Europe, substantial improvements to childhood cancer survival have been identified, with the EUROCARE-6 study estimating age standardised 5-year cancer survival at 81%. This significant progress can be attributed to a combination of achieving earlier cancer diagnosis and improvements to cancer treatment. With almost 500,000 children and adolescent cancer patients across Europe now considered long-term cancer survivors, a rapidly growing cohort of paediatric cancer survivors is becoming established (Botta et al., 2022).

The long-term health of these patients has been actively monitored, with cardiovascular disease identified as a serious complication of anthracycline chemotherapy, which threatens to limit the long-term survival of paediatric cancer survivors (Oeffinger et al., 2006, Haddy et al., 2016). Systematic review analysis estimates that the incidence of anthracycline-induced cardiovascular toxicity could affect up to 57% of paediatric cancer patients treated with anthracycline chemotherapy (Lipshultz et al., 1991, Kremer et al., 2002). Furthermore, retrospective cohort studies have demonstrated that patients are at risk of developing cardiovascular disease well beyond 5-years of cancer-free survival (Mertens et al., 2001, Reulen et al., 2010). Given that anthracyclines are extensively used chemotherapeutic agents, this represents a substantial number of paediatric patients who are at a high risk of morbidity and mortality associated with premature cardiovascular disease (Kremer et al., 2002, Tukenova et al., 2010).

In recognition of these adverse treatment effects, international clinical recommendations have been published as a strategy to ensure all childhood and adolescent cancer survivors receive optimal care, to avert these potentially serious consequences and establish a better quality of survivorship. These guidelines promote early recognition and long-term surveillance of cardiotoxicity, establish the appropriate risk factors, provide guidance on the management of late anthracycline-induced effects, and seek to support patient education (Kremer et al., 2013, Armenian et al., 2015).

5.1.5 Concept of reverse cardio-oncology

To date the main overlap between oncology and cardiology has been upon the impact of malignancy and its treatment upon the heart, but evidence is now coming to light regarding the converse, and the risk of cancer in patients with cardiovascular disease (Aboumsallem et al., 2020, Koelwyn et al., 2022). This is a concept termed 'reverse cardio-oncology', to address the hypothesis that cardiovascular disease has cancer promoting effects, through the combination of shared risk factors, cardiovascular induced immune and inflammatory responses (Aboumsallem et al., 2020, Koelwyn et al., 2022). Although in its infancy, this concept is gaining traction and the requirement for analyses in this context are thus required.

5.1.6 Aims and objectives

Until relatively recently, cardiovascular risk profiles were not routinely screened for and systematically assessed within oncology or haematology patients. Together, cardiologists and oncologists have helped to raise awareness of the impact of cardiovascular disease upon the survival of patients with cancer, given the number of risk factors that are common to both cardiovascular disease and cancer e.g. smoking, age, obesity, physical inactivity, dyslipidaemia, diabetes. To enhance cardiovascular care of patients with cancer, evidence-based risk assessment can also be evaluated in terms of pre-, during and post-cancer treatment. The aims of this chapter are therefore to evaluate the relationship between clinical factors and the development of anthracycline-induced cardiotoxicity, and to outline recommendations for pre- and post-cancer treatment, aiming to improve long-term outcomes and survival of patients through strong evidence-based research. This will be achieved by delivery of the following objectives:

1. Establish the incidence of late-onset anthracycline-induced cardiotoxicity within adult patients treated with anthracycline chemotherapy for breast cancer
2. Evaluate the relationship between baseline blood pressure and the development of the cardiotoxicity phenotype within adult patients treated with anthracycline chemotherapy for breast cancer
3. Compare the cardiovascular risk profiles of patients within the breast cancer patient cohort to those of patients treated for lung cancer and prostate cancer (two patient cohorts associated with high cardiovascular risk), and evaluate the uptake of primary preventative treatments
4. Compare the late-onset cardiotoxicity outcomes in adult patients to the long-term cardio-oncology outcomes within paediatric cancer survivors, using this analysis to propose long-term cardiotoxicity surveillance strategies
5. Explore the concept of reverse cardio-oncology by evaluating the relationship between cardiovascular risk and breast cancer recurrence

5.2 Results

5.2.1 Analysis of the incidence and clinical characteristics of late-onset anthracycline-induced cardiotoxicity within adult patients treated with anthracycline chemotherapy for breast cancer

Patients diagnosed with breast cancer (n=289) and previously recruited to pharmacogenetics studies were retrospectively evaluated for anthracycline-induced cardiotoxicity, with cardiotoxicity defined by a reduction in LVEF to below 55% on echo, and a greater than 10% reduction in LVEF from baseline. As detailed in section 2.13, patients were excluded from the analysis if there was no history of an echocardiogram determining the cardiotoxicity phenotype. Therefore, 81 patients were eligible for inclusion and divided according to known cardiotoxicity status, with baseline characteristics and cardiovascular risk profiles relevant to anthracycline-induced cardiotoxicity presented in **Table 5.2** (Cardinale et al., 2015). No statistical differences between the cardiotoxicity and non-cardiotoxicity cohorts were identified at baseline, aside from concurrent Herceptin treatment, as more patients within the cardiotoxicity group received Herceptin chemotherapy, compared to those without cardiotoxicity (37.9% vs 13.5%, p=0.02). In accordance with international guidelines for the treatment of anthracycline-induced cardiotoxicity, 21 patients out of the 29 diagnosed with the cardiotoxicity phenotype (72.4%) were receiving either an ACEi or ARB, whilst 16 patients of the 29 cardiotoxicity patients (55.2%) were receiving beta-blocker therapy at the time of study analysis.

Within this patient cohort treated with anthracycline chemotherapy for breast cancer, estimated 10-year cardiovascular risk scores (risk of having a heart attack or stroke) for each patient were calculated using the QRISK®3 (https://www.qrisk.org) algorithm. This risk score incorporates known cardiovascular risk factors (i.e. age, gender, diabetes, smoking status, hypertension, cholesterol, body mass index) to provide a percentage risk estimate relevant for the UK population. Comparisons between the 10-year risk scores of patients with cardiotoxicity and without cardiotoxicity are represented in **Table 5.2** and detailed in **Figure 5.1**. Although cardiotoxicity patients were found to have a higher 10-year cardiovascular risk

score (20.2%) compared to patients without cardiotoxicity (16.2%), this difference was not statistically significant (p=0.099).

Table 5.2 **Baseline and clinical characteristics of 89 patients diagnosed with breast cancer and grouped according to anthracycline-induced cardiotoxicity status.** Patients were retrospectively identified from previous anthracycline pharmacogenetics cohorts, having received anthracycline chemotherapy for treatment of their breast cancer, with echocardiogram results used to identify the cardiotoxicity phenotype prior to analysis

Variable	Cardiotoxicity N= 29 (35.8%)	No Cardiotoxicity N= 52 (64.2%)	P-value
Age at time of starting chemotherapy, y (mean ±SD)	57.8 ± 7.0	54.4 ± 9.4	0.085
Age at time of follow-up, y (mean ±SD)	73.9 ± 6.6	71.8 ± 9.3	0.277
Cumulative anthracycline dose, mg/m² *	426.6 ± 72.6	450.8 ± 93.9	0.23
LVEF on echo, n (%): - **Mild** - **Moderate** - **Severe**	 18 (62.1%) 5 (17.2%) 6 (31.6%)	 1 (2.2%) ** 0 (0%) 1 (2.2%) **	 -
Hypertension, n (%)	11 (37.9%)	18 (34.6%)	0.765
Diabetes mellitus, n (%)	6 (20.7%)	11 (21.2%)	0.961
Hypercholesterolaemia, n (%)	1 (3.4%)	6 (11.5%)	0.214
Ischaemic heart disease, n (%)	3 (10.3%)	7 (13.5%)	0.683
Atrial fibrillation, n (%)	4 (13.8%)	8 (15.4%)	0.847
Body mass index, kg/m² (mean ±SD)	28.0 ± 5.7	29.6 ± 6.6	0.328
EGFR (mL/min/1.73m²) (mean ±SD) $	73.6 ± 13.1	75.0 ± 12.5	0.684
Oncology schedule, n (%): - **AC** - **FEC** - **EC**	 25 (86.2%) 2 (6.9%) 2 (69%)	 47 (90.4%) 5 (9.6%) -	 0.566 - -

Variable	Cardiotoxicity N= 29 (35.8%)	No Cardiotoxicity N= 52 (64.2%)	P-value
Adjuvant Trastuzumab, n (%)	11 (37.9%)	7 (13.5%)	0.02
Mediastinal radiotherapy, n (%)	20 (69.0%)	33 (63.5%)	0.743
10-year Cardiovascular risk score (Q-Risk) mean ± SD (%) ¶	20.2 ± 11.1	16.2 ± 11.5	0.099
Breast cancer recurrence	9 (31.0%)	15 (28.8%)	0.84
Mortality at time of analysis, n (%): - **Breast cancer** - **Cardiovascular** - **Other**	 4 (13.8%) 0 (0%) 3 (10.3%)	 6 (11.5%) 1 (2.2%) 11 (21.2%)	 0.767 0.642 0.131 (Combined = 0.328)

Data are expressed as numbers (%) or mean ± SD; P values were calculated using Chi-squared or Fischer's exact test for categorical variables, and using the unpaired t-test for continuous variables. AC indicates doxorubicin, cyclophosphamide; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; EC, epirubicin, cyclophosphamide. LVEF, left ventricular ejection fraction; EGFR, estimated glomerular filtration rate; SD, standard deviation.
* Cumulative anthracycline dose was calculated by converting the anthracycline agents into doxorubicin equivalents.
$ Calculated by Cockcroft-Gault formula
¶ 10-year cardiovascular risk estimated using QRISK®3 (https://www.qrisk.org)
** Left ventricular impairment secondary to myocardial infarction

Statistical analysis for the patient characteristics was completed, with no statistical differences in the establish risk factors calculated between cardiotoxicity and non-cardiotoxicity patient groups. There was no statistical difference between the cardiovascular risk scores, calculated using QRISK3®, of patients diagnosed with cardiotoxicity and without cardiotoxicity (**Figure 5.1**).

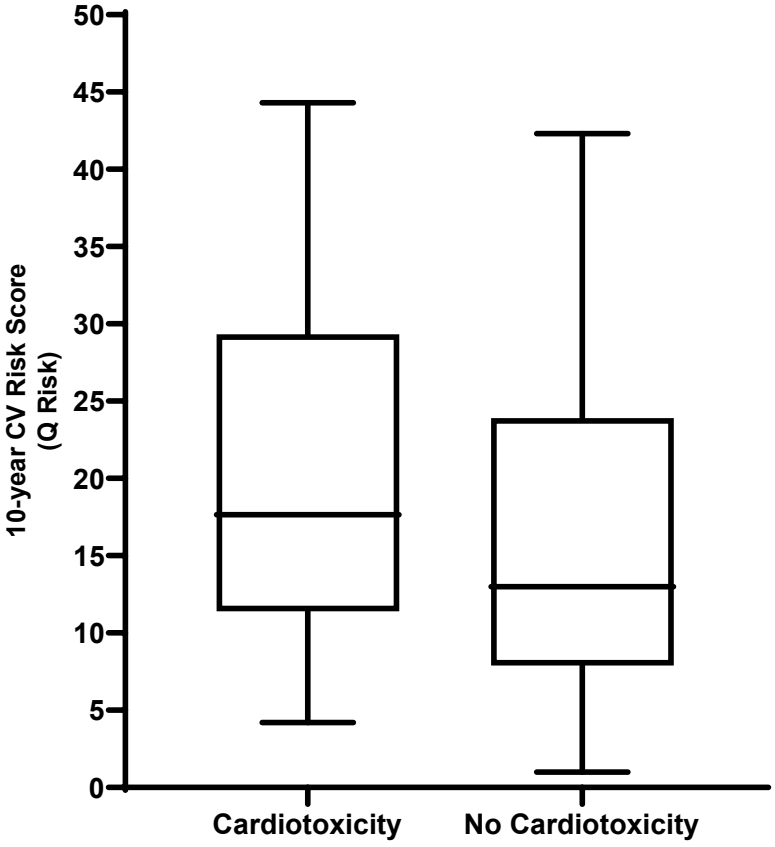


Figure 5.1 **Comparison of estimated 10-year cardiovascular risk scores (determined by QRISK3 calculations) between patients with and without the cardiotoxicity phenotype previous treated with anthracycline chemotherapy for breast cancer. Although patients with cardiotoxicity demonstrate a higher 10-year cardiovascular risk score (20.2%), compared to patients without cardiotoxicity (16.2%),** these results were not found to be statistically significant (p=0.099).

5.2.2 Evaluating the relationship between baseline blood pressure and the development of anthracycline-induced cardiotoxicity

The relationship between baseline blood pressure and the development of anthracycline-induced cardiotoxicity was analysed within the breast cancer patient cohort of this study. Based upon eligibility criteria (section 2.13), thirteen patients were identified for inclusion within the study. Of these thirteen patients, six patients had developed cardiotoxicity (46.2%). Three of these patients presented with mild left ventricular dysfunction (LVEF: 45-55%), and three presented with severe left ventricular dysfunction (LVEF: ≤35%). The patient characteristics are presented in (**Table 5.3**).

Mean baseline systolic blood pressure measurements for the patients developing cardiotoxicity was 138.2 ± 13.8 mmHg, compared to 131.4 ± 16.8 mmHg for those patients with no reported cardiotoxicity or decline in LVEF. Mean baseline diastolic blood pressure measurements in patients identified with cardiotoxicity was 85.2 ± 6.0 mmHg compared to 74.3 ± 5.4 mmHg in non-cardiotoxic patients. Levene's Test for Equality of Variances demonstrated equal variances can be assumed for both populations (Systolic: $F = 0.308$, $p = 0.590$; Diastolic: $F = 0.004$, $p = 0.953$). No significant association ($p = 0.450$) was identified between baseline systolic blood pressure and development of cardiotoxicity (**Figure 5.2**). In contrast, baseline diastolic blood pressure demonstrated a statistically significant relationship ($p=0.005$) with development of anthracycline-associated cardiotoxicity, defined by a reduction in LVEF (**Figure 5.2**).

Table 5.3 **Characteristics of breast cancer patients treated with doxorubicin.** Cardiotoxicity defined by decline in left ventricular ejection fraction (LVEF), as measured at echocardiographic follow-up. Baseline blood pressures recorded within 12 months prior to initiation of chemotherapy. ± SD: standard deviation; [ ] CI: confidence intervals.

	Patients with cardiotoxicity	**Patients without cardiotoxicity**
Number of cases	6	7
Age at treatment	57.7 ± 4.9 years	55.3 ± 7.8 years
Body surface area (mean ± SD)	1.74 ± 0.07 m^2	1.88 ± 0.22 m^2
Total dose received (mean ± SD)	382.2 ± 84.8 mg/m^2	450.7 ± 54.0 mg/m^2
Clinical follow-up period (median [*95% CI*])	15.5 [*12.0-17.5*] years	17.0 [*9.0-19.0*] years
Cases with mild cardiotoxicity [LVEF: 45-55%]	3	-
Cases with severe cardiotoxicity [LVEF: ≤35%]	3	-
Pre-treatment timing of baseline blood pressure measurement (median [*95% CI*])	5 [*1.5-6.5*] months	7 [*1.5-10*] months
Baseline systolic blood pressure (mean ± SD)	138.2 ± 13.8 mmHg	131.4 ± 16.8 mmHg
Baseline diastolic blood pressure (mean ± SD)	85.2 ± 6.0 mmHg	74.3 ± 5.4 mmHg

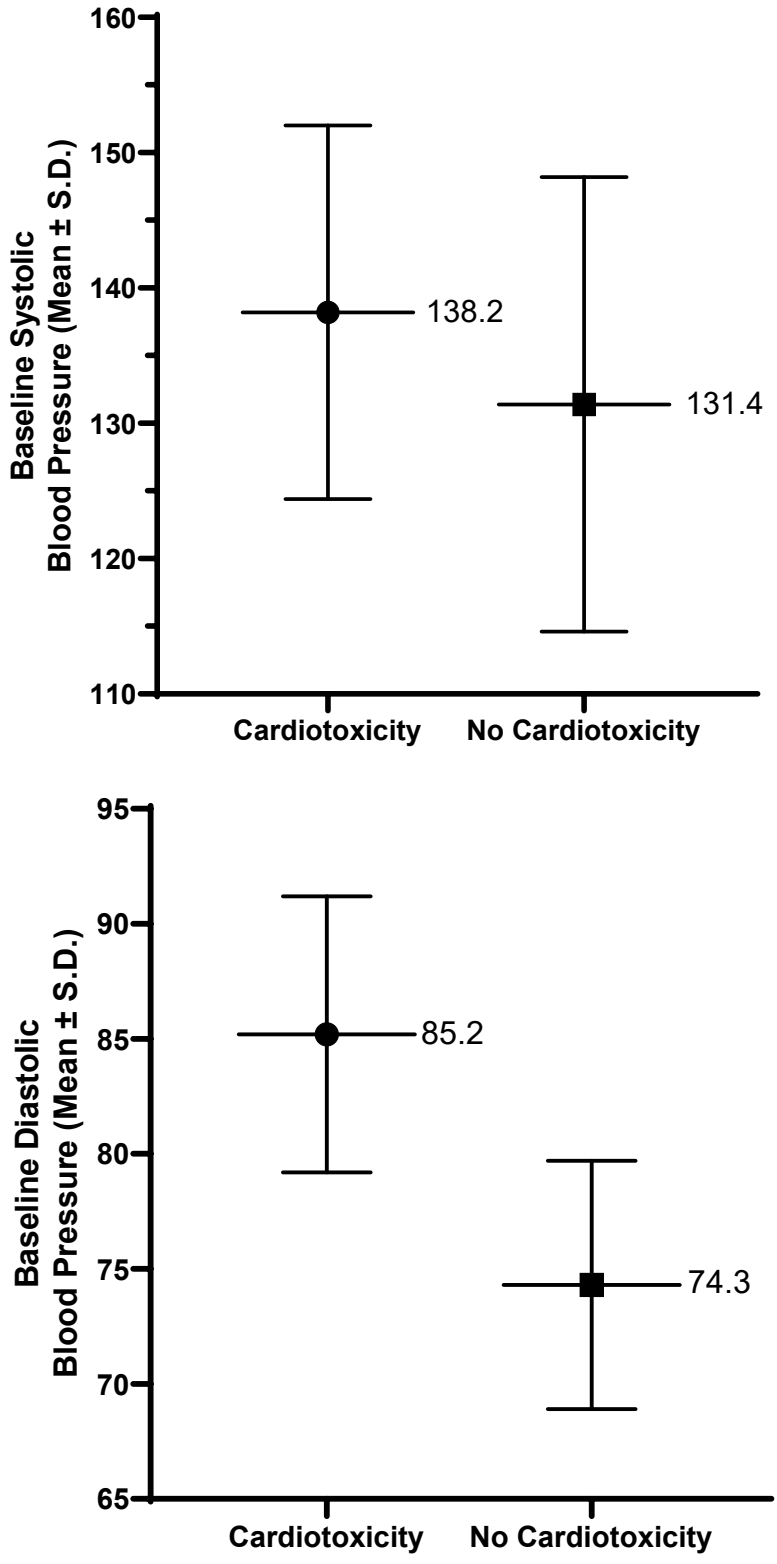


Figure 5.2 **Retrospective analysis of mean baseline blood pressures in breast cancer patients presenting with and without cardiotoxicity.** Elevated baseline diastolic, but not systolic, blood pressure correlates with development of anthracycline-associated cardiotoxicity. Cardiotoxicity as defined by a reduction in LVEF (<55%) and >10% from baseline.

5.2.3 Analysis of the cardiovascular risk profiles in patients treated for breast cancer

The cardiovascular risk profiles of patients (n=208 females) diagnosed and previously treated for breast cancer were evaluated (see section 2.13). Their individual cardiovascular risk factors were evaluated and collated to calculate their 10-year cardiovascular risk using the QRISK3® algorithm, whilst medications were reviewed for lipid lowering therapy in accordance with National Institute for Health and Clinical Excellence (NICE) clinical guidelines [CG181: September 2016] (see section 2.14). The results are presented in **Figure 5.3** and **Figure 5.4**.

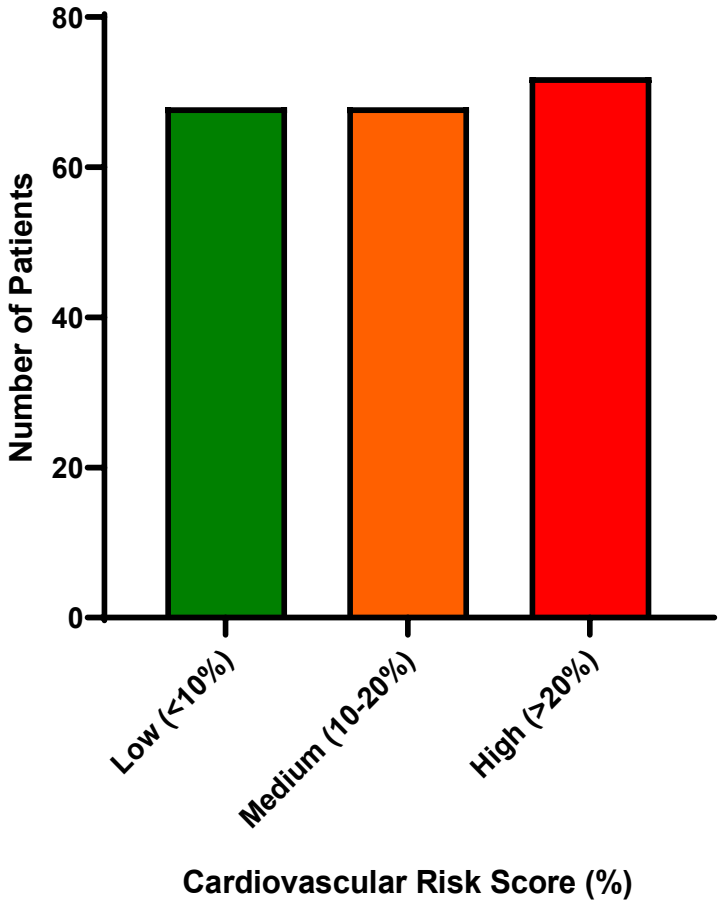


Figure 5.3 **Cardiovascular risk stratification of patients with breast cancer calculated using QRISK3® risk calculator**. Patients are grouped according to low (<10%), medium (10-20%) and high (>20%) cardiovascular risk.

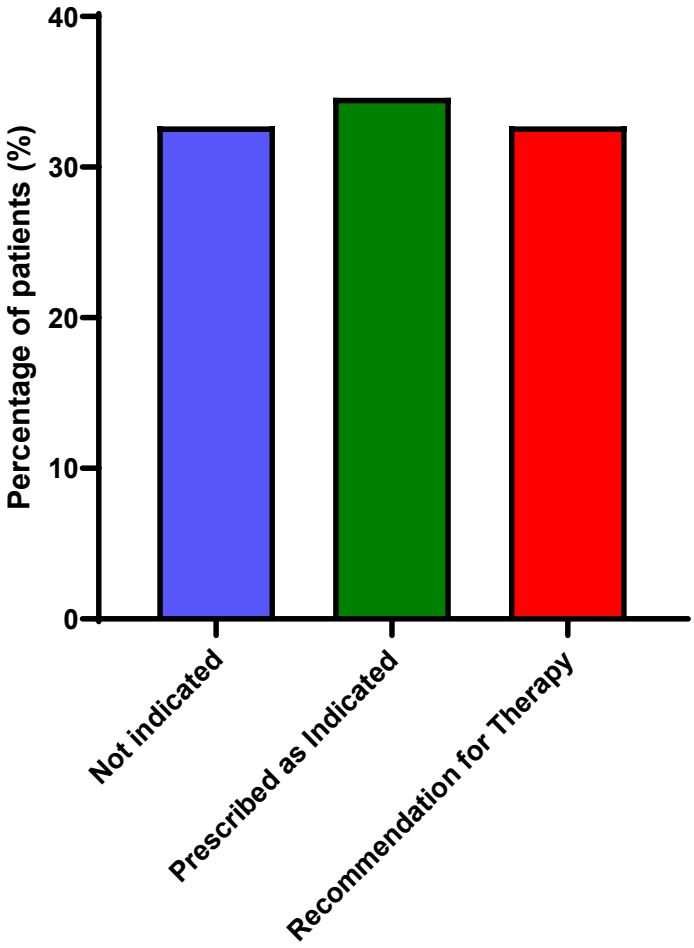


Figure 5.4 **Statin therapy status of patients with breast cancer following cardiovascular risk assessment using QRISK3 and in accordance with NICE lipid modification guidelines**

This analysis demonstrates that from 208 breast cancer survivors, 72 patients (34.4%) were categorised as having a high cardiovascular risk (QRISK3® score >20%) meaning that their risk of developing a cardiovascular event (i.e. heart attack or stroke) over the next 10 years is greater than 20%. 68 patients (32.5%) were found to be of medium cardiovascular risk (QRISK3® score 10-20%), whilst 68 patients (32.5%) were of low cardiovascular risk (QRISK3® score <10%).

The results of the lipid lowering therapy analysis demonstrated that of 140 patients eligible for oral statin therapy as recommended by the NICE lipid modification guidelines [CG181], 72 patients (51.4%) were prescribed at statin or alternative cholesterol lowering therapy, whilst 68 patients (48.6%) were eligible for statin therapy but not prescribed this at the time of the analysis.

5.2.4 Analysis of the cardiovascular risk profiles in patients treated for prostate cancer

The cardiovascular risk profiles of patients diagnosed and receiving treatment for prostate cancer were also evaluated (n=20), with inclusion criteria detailed in section 2.14. Of the twenty men included within the study, nineteen had received hormone therapy for localised prostate cancer, whilst one patient was receiving treatment for metastatic prostate cancer. As with the breast cancer cohort, individual cardiovascular risk factors were assessed and 10-year cardiovascular risk using the QRISK3® algorithm was calculated, with lipid-lowering therapy status evaluated (**Figure 5.5)**.

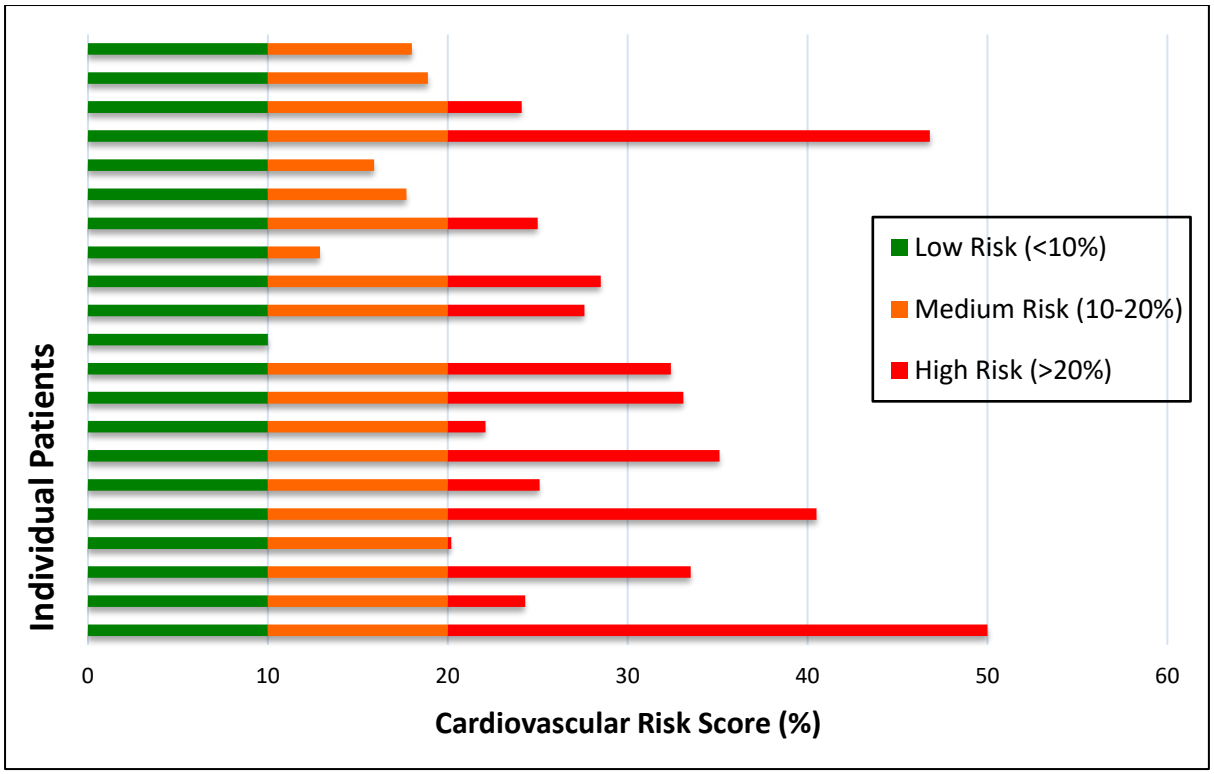


Figure 5.5 Cardiovascular risk stratification of patients with prostate cancer calculated using QRISK®3 risk calculator

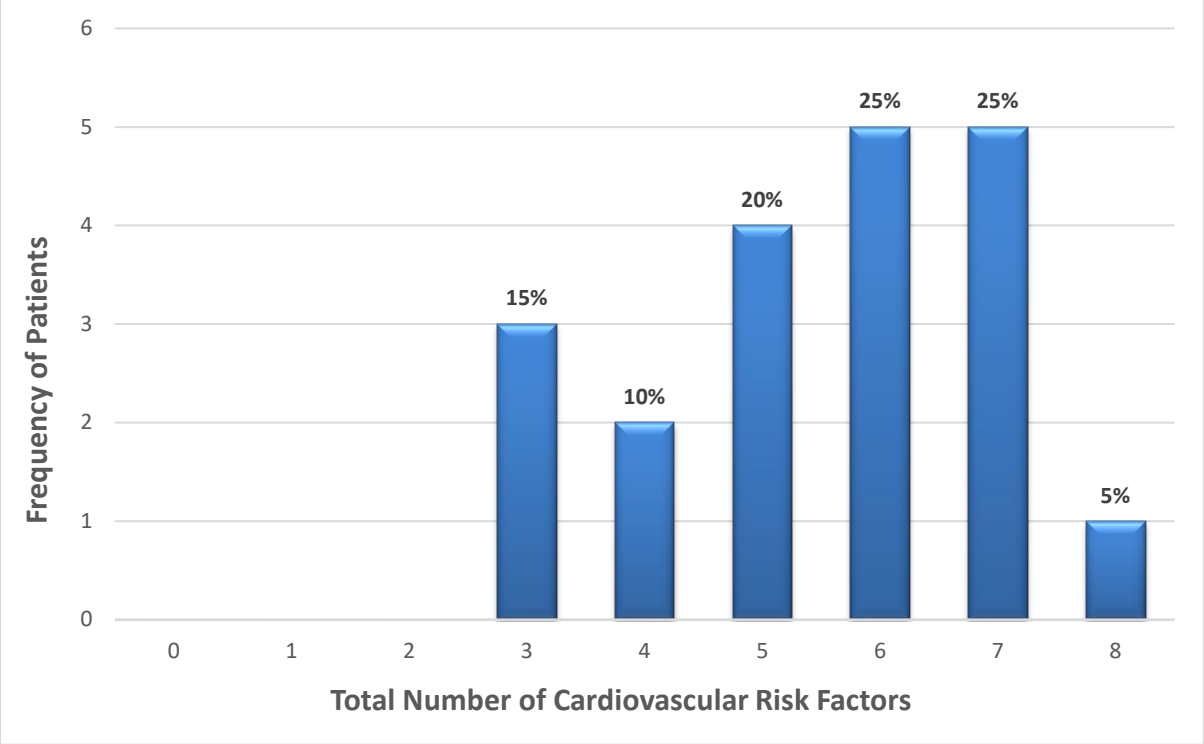


Figure 5.6 **The frequency of established cardiovascular risk factors amongst patients with diagnosed prostate cancer attending outpatient urology clinics**

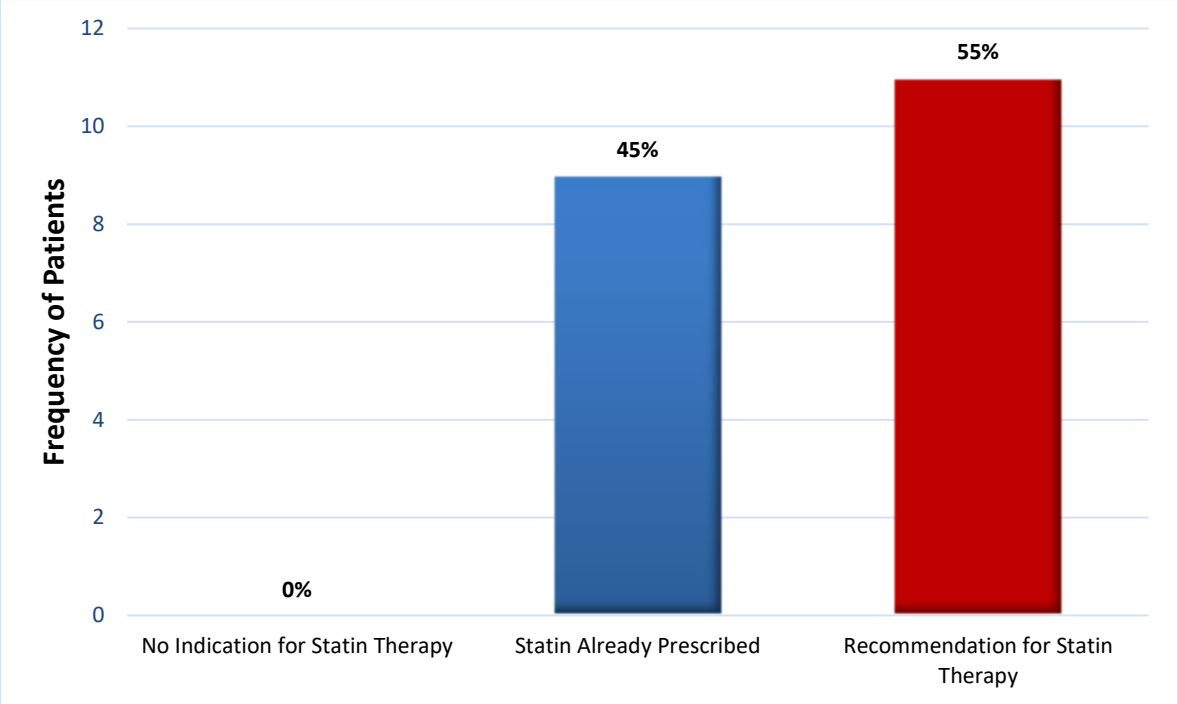


Figure 5.7 **Statin therapy status of patients with prostate cancer attending outpatient clinics following cardiovascular risk assessment and review of NICE guideline recommendations for statin therapy**

The results from this prostate cancer cohort demonstrated that of the 20 patients included, 15 patients (75%) were classified has high cardiovascular risk (10-year risk of a stroke or heart attach >20% as calculated by QRISK3®), whilst 5 patients (25%) were recognised as medium cardiovascular risk (10-20%). No patients were identified as low cardiovascular risk according the QRISK3® algorithm (10-year risk <10%) (**Figure 5.5**).

All 20 patients within the study were therefore eligible for consideration of statin therapy recommended by the NICE guidelines and QRISK3® cardiovascular risk score. Of these 20 patients, 9 patients (45%) were already prescribed statin therapy, whilst 11 patients (55%) although eligible for therapy were not taking a statin or alternative cholesterol-lowering agent at the time of analysis (**Figure 5.7**).

5.2.5 Analysis of the cardiovascular risk profiles in patients treated for non-small cell lung cancer (NSCLC)

The cardiovascular risk profiles of patients diagnosed and receiving treatment for lung cancer were also evaluated (n=20), with inclusion criteria detailed in section 2.14. This analysis evaluated fourteen females (70%) and six males (30%) lung cancer patients. Their cardiovascular risk factors and 10-year cardiovascular risk scores were calculated using QRISK3® in addition to lipid lowering therapy status (**Figure 5.8**).

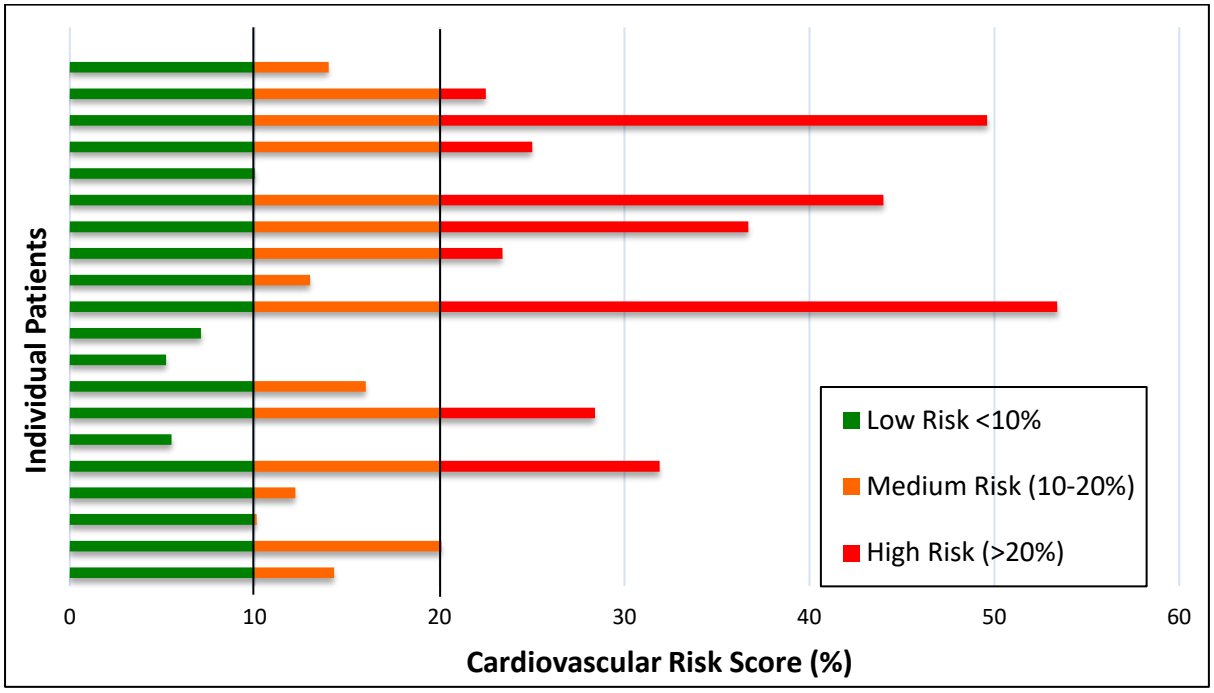


Figure 5.8 **Cardiovascular risk stratification of patients with lung cancer calculated using QRISK®3 risk calculator**

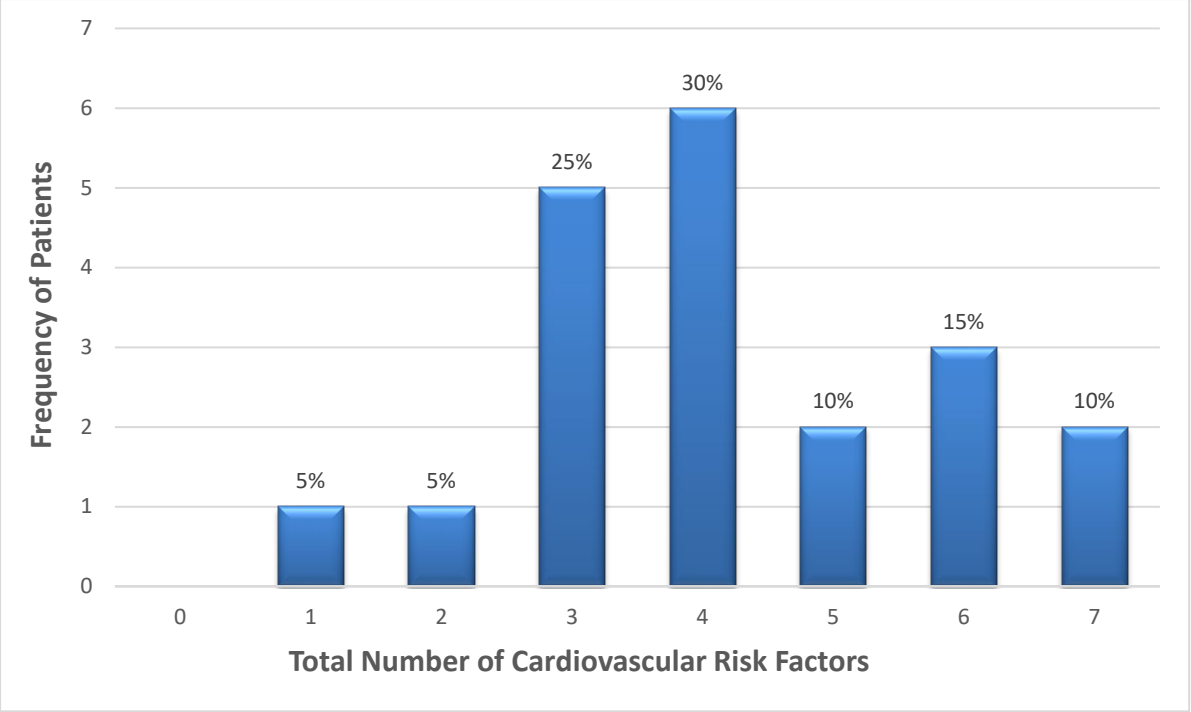


Figure 5.9 **The frequency of established cardiovascular risk factors amongst patients with diagnosed lung cancer attending outpatient respiratory clinics**

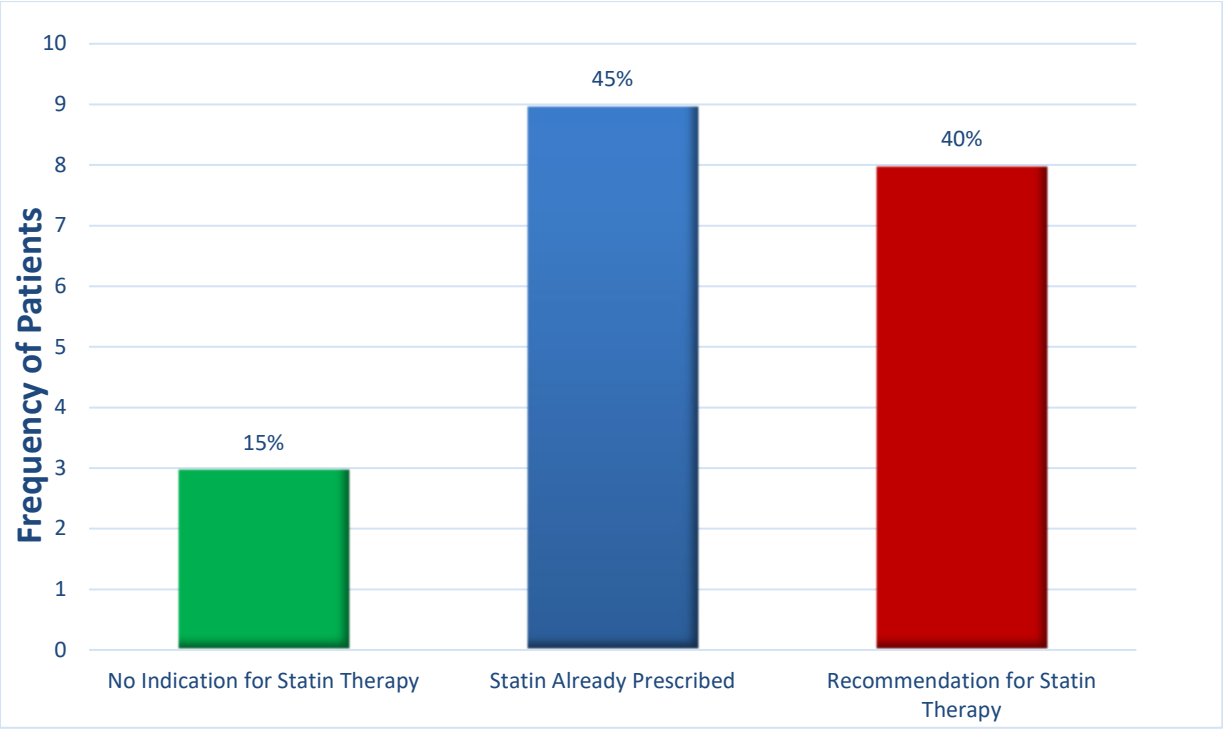


Figure 5.10 **Statin therapy status of patients with lung cancer attending outpatient clinics following cardiovascular risk assessment and review of NICE guideline recommendations for statin therapy**

The results of the lung cancer patient cohort demonstrated that of the 20 patients included within the analysis, 10 patients (50%) were identified as high cardiovascular risk (QRISK3® score >20%), whilst 7 patients (35%) were medium risk (10-20%), and 3 patients (15%) were considered low cardiovascular risk (i.e. 10-year risk of a cardiovascular event <10%) (**Figure 5.8**).

Lipid-lowering therapy was therefore not indicated in these low cardiovascular risk patients (<10%). Where statin therapy was indicated in the 17 patients considered either medium or high cardiovascular risk, 9 patients (52.9%) were prescribed statin therapy, whilst 8 patients (47.1%) although eligible and statin therapy indicate, were not prescribed lipid-lowering therapy for cardiovascular risk modification (**Figure 5.10**).

5.2.6 Analysing the long-term incidence of anthracycline-induced cardiotoxicity within adult patients

Within the breast cancer pharmacogenetics patient cohort, 81 patients of 289 patients had been evaluated for cardiotoxicity since receiving anthracycline chemotherapy treatment using echocardiography (**Table 5.2**). Statistically significant differences between the total pharmacogenetic cohort (289 patients) and the cohort of adult patients with breast cancer (81 patients), with the 81-patient cohort demonstrating a higher proportion of patients with diabetes mellitus (21.0% vs. 11.1%, p=0.03), ischaemic heart disease (12.3% vs. 3.8%, p=0.004), atrial fibrillation (14.8% vs. 7.3%, p=0.035), and having received concurrent Herceptin chemotherapy (22.2% vs. 10.7%, p=0.007). Otherwise, the baseline characteristics, as set out in **Table 5.2**, were similar between the two cohorts.

The incidence of cardiotoxicity within the breast cancer pharmacokinetics cohort was calculated following independent review by two cardiologists, with 29 patients (35.8%) diagnosed with cardiotoxicity and 52 patients (64.2%) without cardiotoxicity. The median duration of patient follow-up for cardiotoxicity since completing chemotherapy was 18 years, with all patients followed up for at least 10 years.

The cumulative incidence of cardiotoxicity was demonstrated using Kaplan Meier curve (**Figure 5.11**), analysing the time from completion of anthracycline chemotherapy until diagnosis of anthracycline-induced cardiotoxicity. From the 29 cardiotoxicity cases, 1 patient (3.4%) developed cardiotoxicity during anthracycline treatment, 7 patients (24.1%) were

diagnosed with cardiotoxicity within one year of chemotherapy completion, whilst 21 patients (72.4%) developed anthracycline-induced cardiotoxicity after one year. The mean time to diagnosis from chemotherapy completion was 8.3 years, and the median 8.7 years (interquartile range, 1 – 14.1 years), whilst the maximum duration to development of cardiotoxicity was found to be 18.9 years.

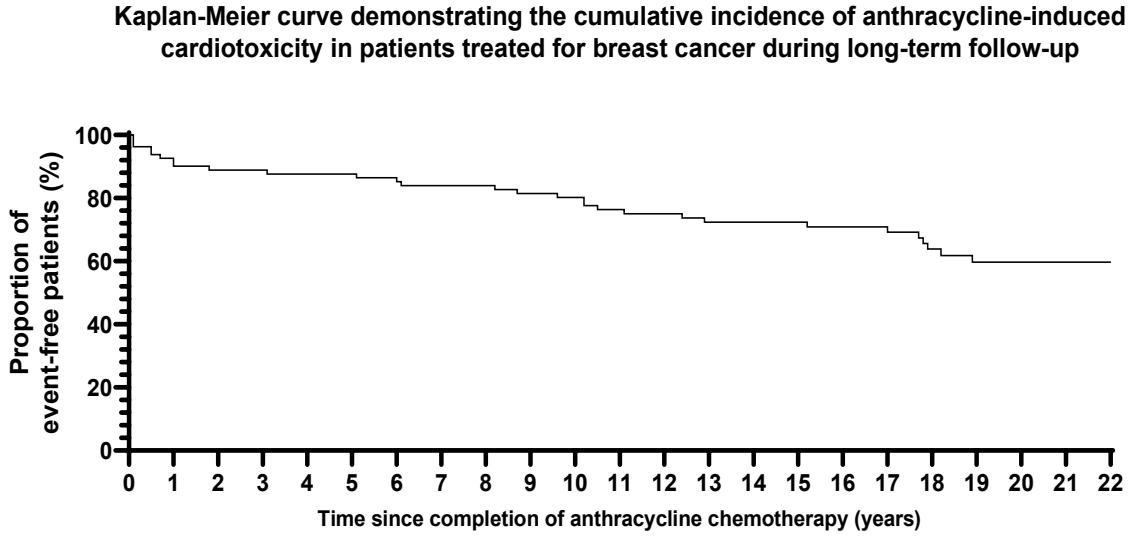


Figure 5.11 Kaplan-Meier curve demonstrating the cumulative incidence of anthracycline-induced cardiotoxicity in patients treated for breast cancer during long-term follow-up

Of the 29 patients diagnosed with anthracycline-induced cardiotoxicity, ACE inhibitor or ARB therapy was initiated in 21 patients (72.4%), with 16 patients (55.2%) receiving concurrent betablocker therapy. There were no recorded cardiovascular or cardiotoxicity-related deaths within the breast cancer patient cohort.

5.2.7 Analysing the long-term incidence of anthracycline-induced cardiotoxicity in paediatric cancer survivors

Patients (n=173; mean age at follow-up 18.1 ± 5.4 years, 50.3% females, 49.7% males) who had received anthracycline based therapy as children, were evaluated in this study, with a median follow-up duration post-anthracycline chemotherapy of 9.3 years (interquartile range (IQR), 7.7 – 12.9 years; range, 5.3 – 26.3 years). All patients were followed up for more than 5 years to evaluate for cardiotoxicity, whilst 42.3% were followed up for more than 10 years.

Cardiotoxicity was identified in 41 (23.7%) patients, whilst 132 (76.3%) patients had no evidence of cardiotoxicity on echocardiogram. The patient characteristics are evaluated in **Table 5.4**, with patients grouped according to cardiotoxicity status (Cardinale et al., 2015).

Table 5.4 Patient characteristics according to cardiotoxicity status within long-term childhood cancer survivors

Variable	Cardiotoxicity n=41 (23.7%)	No Cardiotoxicity n= 132 (76.3%)	P-value
Age at time of primary diagnosis, y	7.4 ± 5.4	5.3 ± 4.4	0.01
Age at time of completing chemotherapy, y	8.9 ± 5.7	7.1 ± 4.2	0.03
Age at time of follow-up, y	22.6 ± 5.4	16.7 ± 4.6	<0.001
Female sex, n (%)	19 (46.3%)	68 (51.5%)	0.563
Cumulative anthracycline dose, mg/m^2	315.8 ± 162.7	192.9 ± 99.6	< 0.001
Anthracycline, n (%): - Doxorubicin *Dose (mg/m^2)*	18 (43.9%) 231.9 ± 121.5	81 (61.4%) 175.3 ± 100.5	0.08
- Daunorubicin *Doxorubicin Equivalent Dose (mg/m^2)*	8 (19.5%) 253.8 ± 47.1	23 (17.4%) 193.6 ± 107.3	0.04
- Daunorubicin & Doxorubicin *Doxorubicin Equivalent Dose (mg/m^2)*	3 (7.3%) 233 ± 0	14 (10.6%) 205 ± 36.6	0.0135
- Daunorubicin & Epirubicin *Doxorubicin Equivalent Dose (mg/m^2)*	0	1 (0.8%) 284	-
- Daunorubicin & Idarubicin *Doxorubicin Equivalent Dose (mg/m^2)*	0	7 (5.3%) *275.6 ± 84.2*	-
- Daunorubicin & Mitoxantrone *Doxorubicin Equivalent Dose (mg/m^2)*	12 (29.2%) *503.6 ± 131.9*	2 (1.5%) *329 ± 171.1*	*0.3699*
- Doxorubicin & Mitoxantrone *Doxorubicin Equivalent Dose (mg/m^2)*	0	1 (0.8%) *230*	-
- Idarubicin & Mitoxantrone *Doxorubicin Equivalent Dose (mg/m^2)*	0	1 (0.8%) *226*	-

Variable	Cardiotoxicity n=41 (23.7%)	No Cardiotoxicity n= 132 (76.3%)	P-value
- Doxorubicin, Daunorubicin & Mitoxantrone *Doxorubicin Equivalent Dose (mg/m^2)*	0	2 (1.5%) 309 ± 5.7	- -
Cumulative dose anthracyclines: - Low dose (<250 mg/m^2) - High dose (≥ 250 mg/m^2)	 16 (14.4%) 25 (40%)	 95 (85.6%) 37 (59.7%)	 < 0.001
Bone Marrow Transplant, n (%)	16 (39.0%)	17 (12.9%)	< 0.001
Stem Cell Transplant, n (%)	4 (9.8%)	7 (5.3%)	0.292
Mediastinal radiotherapy, n (%)	4 (9.8%)	12 (9.1%)	0.898
Total Body Radiotherapy, n (%) *Dose (Gray, Gy)*	10 (24.4%) *15 ± 1.9*	8 (6.1%) *13.9 ± 1.2*	0.002 *0.144*
Oncology Diagnosis, n (%): - ALL - AML - Leukaemia, other - Hodgkin's Lymphoma - Non-Hodgkin's Lymphoma - Myelodysplasia - Wilm's Tumour - Sarcoma - Rhabdomyosarcoma - Neuroblastoma - Hepatoblastoma - Retinoblastoma - Histiocytoma - Adrenocortical - Renal Cell	 9 (22.0%) 15 (36.6%) 1 (2.4%) 1 (2.4%) 2 (4.9%) 2 (4.9%) 3 (7.3%) 4 (9.8%) 2 (4.9%) 2 (4.9%) 0 0 0 0 0	 66 (50%) 19 (14.4%) 2 (1.5%) 7 (5.3%) 11 (8.3%) 0 7 (5.3%) 8 (6.1%) 1 (0.8%) 3 (2.3%) 3 (2.3%) 1 (0.8%) 1 (0.8%) 1 (0.8%) 2 (1.5%)	-

The Kaplan-Meier curve (**Figure 5.12**) illustrates the cumulative incidence of cardiotoxicity, with time taken from completion of chemotherapy to developing anthracycline cardiotoxicity. The mean time for cardiotoxicity development in this cohort was 5.5 ± 5.6 years, with the median time 4.1 years (IQR, 0.2 – 9.2 years), whilst the overall range was from during treatment up to 19.1 years since chemotherapy completion. Of the patients diagnosed with cardiotoxicity, 9 patients (22.0%) established acute cardiotoxicity (during anthracycline treatment), whilst 5 patients (12.2%) developed sub-acute cardiotoxicity (within year after chemotherapy completion). The remaining 27 (65.9%) patients were diagnosed with delayed chronic cardiotoxicity (>1 year after treatment), of which 20 patients (48.8%) were diagnosed with cardiotoxicity after 5 years since completion of chemotherapy treatment. Comparison of this data within paediatric patients is made Cardinale et al. (2015), in which their Kaplan-Meier curve (**Figure 5.13**) illustrates the incidence of anthracycline-induced cardiotoxicity within adult patients. In contrast to the paediatric data, 98% of cardiotoxicity cases were diagnosed within the first year of follow-up, as illustrated by the steep portion of the curve, with a median of 3.5 months between the last anthracycline dose and detection of cardiotoxicity. There were only 5 patients in the adult study who developed cardiotoxicity from years 1 to 6 post-chemotherapy, and therefore classified as late-onset cardiotoxicity, as represented by the flatting of the curve until study completion (Cardinale et al., 2015).

Of the patients diagnosed with cardiotoxicity within the paediatric study, retrospective analysis demonstrated that heart failure therapy was initiated in 15 (39.5%) patients, with these patients receiving either an ACE inhibitor (36.8%), a beta-blocker (13.2%) or both (10.5%). Within the paediatric cardiotoxicity cohort, 3 patients (7.3%) received a cardiac transplantation for anthracycline-induced cardiotoxicity, and were therefore excluded from the heart failure therapy analysis. No patients were receiving ACEi or betablocker therapy prior to receiving anthracycline chemotherapy.

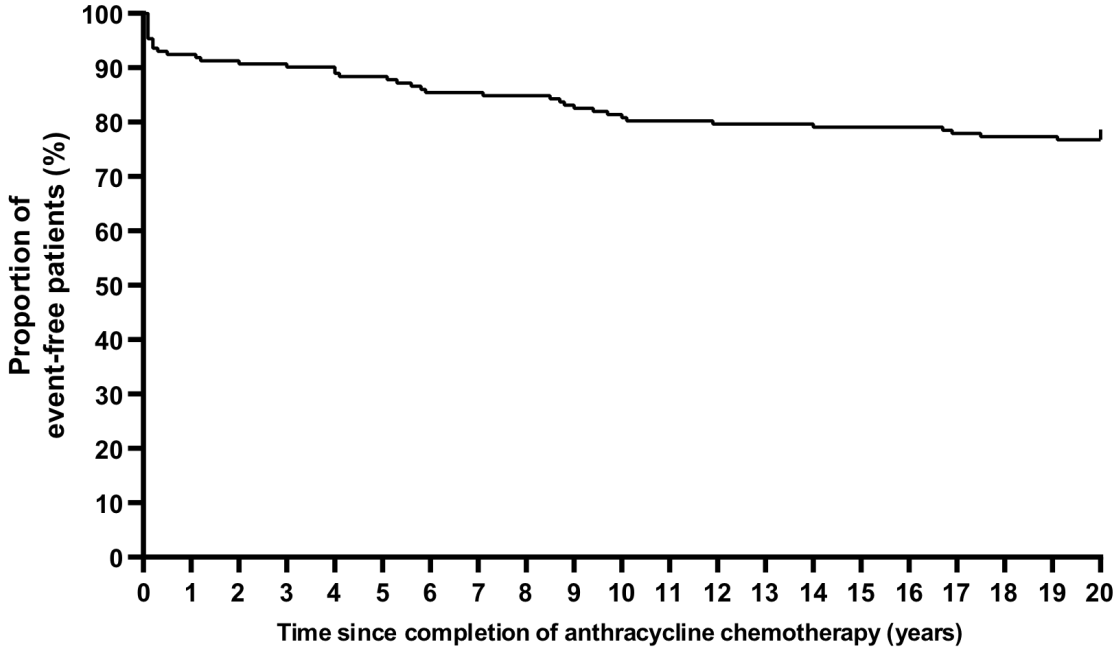


Figure 5.12 **Kaplan-Meier curve demonstrating the cumulative incidence of anthracycline-induced cardiotoxicity in long-term paediatric cancer survivors**

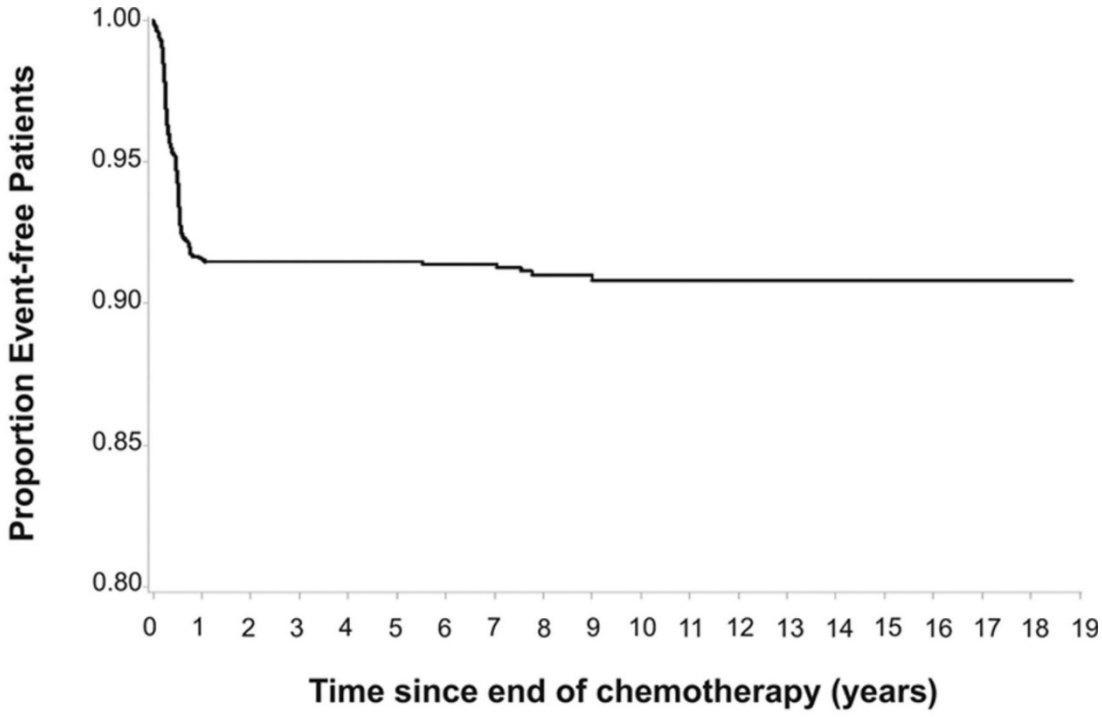


Figure 5.13 **Kaplan-Meier curve taken from Cardinale et al. (2015) showing the cumulative incidence of cardiotoxicity in adult patients**

5.2.7.1 Cox Multiple Regression Analysis

Risk factors for cardiotoxicity were evaluated using Cox multiple regression analysis. Age at treatment completion (hazard ratio [HR], 1.365; 95% confidence interval [CI], 1.017 – 1.832, for every 5-year increase), cumulative anthracycline dose (HR, 1.264; 95% CI, 1.017 – 1.832, for each 50mg/m^2 increase), mediastinal radiotherapy (HR, 0.606; 95% CI, 0.200 – 1.836), and female gender (HR, 1.090; 95% CI, 0.566 – 2.135) were analysed as independent correlators of cardiotoxicity. These results demonstrated that increased age and cumulative anthracycline dose were statistically significant correlators of cardiotoxicity. The statistical analysis in **Table 5.4** found a greater proportion of patients within the cardiotoxicity group had received total body radiotherapy, compared to those without the cardiotoxicity phenotype (24.4% vs. 6.1%, $p<0.002$). However, when total radiotherapy doses were evaluated, no statistical difference between the two groups was identified.

Further analysis was completed to evaluate the International Late Effects of Childhood Cancer Guidelines threshold of cumulative anthracycline dose upon the incidence of cardiotoxicity. These guidelines detail that children and adolescents receiving total anthracycline doses ≥250mg/m^2 are considered at high risk of developing anthracycline-induced cardiotoxicity (Armenian et al., 2015). Therefore, the paediatric patient cohort was analysed according to this threshold, with almost a 4-fold increase in cardiotoxicity events associated with doses ≥250mg/m^2, compared to the doses <250mg/m^2 (HR 3.842; 95% CI 1.858 – 6.526, $p<0.05$) (**Figure 5.14**).

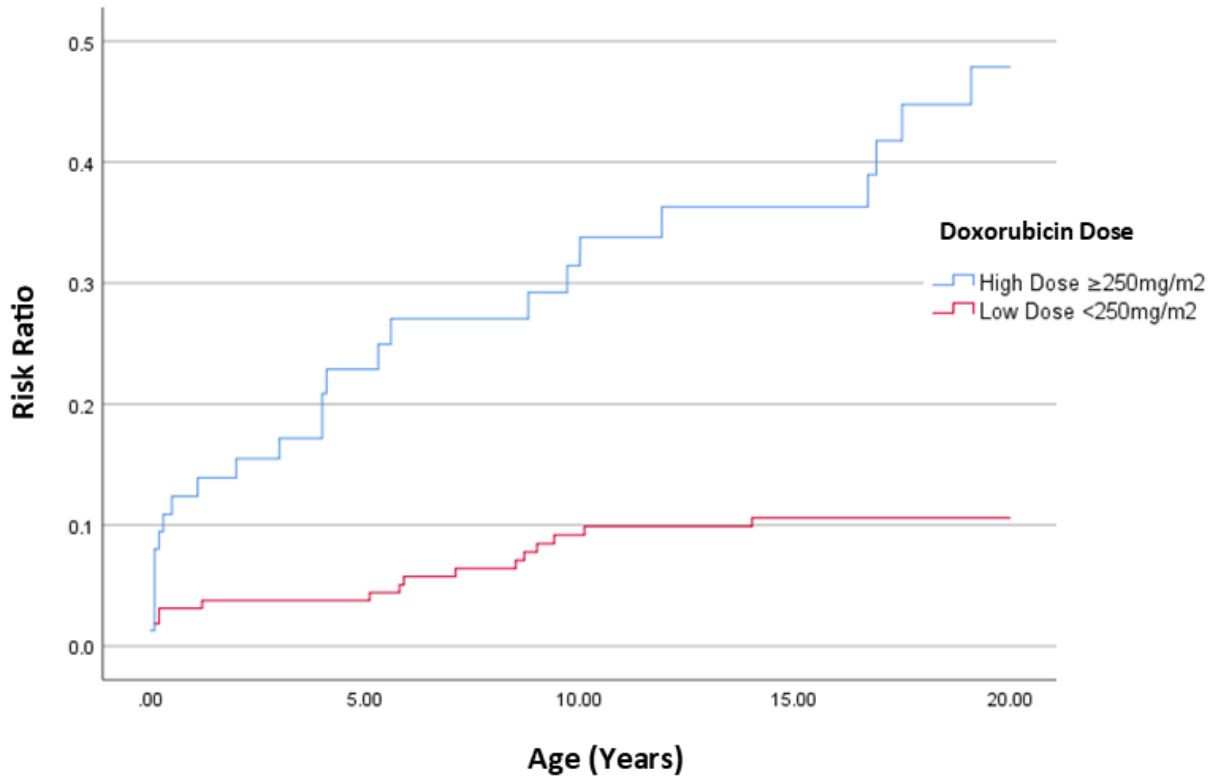


Figure 5.14 **Risk ratio for high versus low cumulative doxorubicin dose for development of cardiotoxicity in paediatric patients**. The blue line indicates the cardiotoxicity incidence in paediatric patients receiving high cumulative doses of anthracyclines ≥250mg/m^2, and the red line indicates the paediatric patient cohort who received lower doses <250mg/m^2. (HR 3.842; 95% CI 1.858 – 6.526, p<0.05).

5.2.7.2 Cardiomyopathy Risk Stratification

Patients identified with anthracycline-induced cardiotoxicity were retrospectively evaluated and stratified according the cardiomyopathy risk stratification (table 1) published by the International Late Effects of Childhood Cancer Guideline Harmonization Group (Armenian et al., 2015). These results (**Table 5.5**) demonstrate that 26 patients (63.4%) with anthracycline-induced cardiotoxicity were classified as high risk, 14 patients (34.1%) identified as moderate risk, and 1 patient (2.4%) as low risk.

Table 5.5 **Cardiomyopathy risk stratification of patients identified with cardiotoxicity and based upon cumulative anthracycline dose and radiotherapy dose exposure**

Low Cardiomyopathy Risk	Moderate Cardiomyopathy Risk	High Cardiomyopathy Risk
1 patient (2.4%)	14 patients (34.1%)	26 patients (63.4%)

5.2.8 Analysing the relationship between cardiovascular risk and breast cancer recurrence

173 adult patients were eligible for inclusion within the study based upon cancer recurrence status. Of this cohort, 10-year cardiovascular risk estimates using QRISK3® were calculated in 154 patients, with 19 patients excluded as they had died since enrolment in the study and thus their electronic records were not accessible for accurate CV risk score calculation, with 5 of these 19 patients having died with recurrent breast cancer. Therefore, of the 154 patients included, 22 patients (14.3%) had a recurrence of their breast cancer, with 132 patients (85.7%) had no evidence of cancer recurrence.

The relationship between cardiovascular risk, as defined by calculated QRISK3 10-year cardiovascular event risk, and breast cancer recurrence was evaluated. Following calculation of their QRISK3® scores, patients were grouped according to low (<10% 10-year cardiovascular event risk), medium (10-20%) and high (>20%). The results are demonstrated in **Table 5.6**.

Table 5.6 The relationship between cardiovascular risk and breast cancer recurrence, with p-values calculated using chi-squared test

QRISK3® risk category	Patients with breast cancer recurrence (n=22)	Patients with no breast cancer recurrence (n=132)	P-value
Low (<10%)	7 (31.8%)	43 (32.6%)	0.944
Medium (10-20%)	8 (36.4%)	41 (31.1%)	0.621
High (>20%)	7 (31.8%)	48 (36.4%)	0.68

This preliminary analysis demonstrated no association between breast cancer recurrence and cardiovascular risk estimation was demonstrated in this analysis, with the proportions of patients (%) identified within each cardiovascular risk group being relatively similar. The association between breast cancer recurrence and cardiotoxicity was also explored (**Table 5.2**). 9 patients with cardiotoxicity (31.0%) compared to 15 patients without cardiotoxicity (28.8%) were diagnosed with a recurrence of breast cancer during follow-up, with no statistically significant difference between the groups detected (p=0.84).

5.3 Discussion

Cancer patients are recognised as having a higher cardiovascular risk than the general population, with cancer survivors who develop cardiovascular disease (CVD) having a worse all-cause mortality than cancer survivors without CVD. In addition to the high cardiovascular risk burden within cancer survivors, cancer therapies including radiotherapy and certain types of chemotherapy are associated with an increased risk of cardiovascular disease and events (Tukenova et al., 2010, van Nimwegen et al., 2015). Therefore, the established principles of cardio-oncology are: firstly, ensuring patients with cancer receive their essential cancer treatment, and secondly, promoting long-term cancer survival through prevention of cardiovascular complications relating to cancer therapies and the optimisation of cardiovascular risk factors. To enhance the cardiovascular care of patients with cancer, evidence-based risk assessment can be evaluated in terms of pre-, during and post-cancer treatment, with the aim of outlining recommendations for pre- and post-cancer treatment, to improve long-term outcomes and survival of patients through strong evidence-based research.

The 2022 European Society of Cardiology (ESC) guidelines on cardio-oncology provide recommendations for cardiovascular toxicity monitoring in patients receiving anthracycline chemotherapy. Through a combination of cardiovascular risk factor assessment, measurement of clinical biomarkers, and echocardiography surveillance, patients are stratified according to their risk of developing chemotherapy-related cardiac dysfunction (Lyon et al., 2022). Based upon prospective data studying 2625 patients diagnosed and treated with anthracycline chemotherapy for breast cancer, clinical recommendations within these guidelines support cardiovascular risk assessment for up to 12 months following the last dose of cardiotoxic chemotherapy (Lyon et al., 2022). This study from Cardinale et al. (2015), found the cumulative incidence of cardiotoxicity within their cohort to be 9%, with 98% of anthracycline-induced cardiotoxicity cases detected within the first 12 months of chemotherapy completion (Cardinale et al., 2015). From empirical evidence within the cardio-oncology clinics, patients treated with anthracycline chemotherapy have presented beyond this 12-month period, with either echocardiography changes or clinical heart failure symptoms consistent with a diagnosis of anthracycline-induced cardiotoxicity. As specified in an earlier study by Cardinale et al. (2010), treatment of anthracycline-induced reductions in

LVEF is crucially time-sensitive. Longer delays to ACEi initiation were associated with a poorer response to heart failure treatment, to the degree that no recovery in left ventricular function was observed if treatment was started beyond 6 months of chemotherapy completion (Cardinale et al., 2010). With these studies driving these guideline recommendations, the long-term incidence of anthracycline-induced cardiotoxicity was evaluated using a historical anthracycline pharmacogenetic cohort of patients treated with anthracyclines for breast cancer. Selection of this patient cohort provides significance within the current literature, as it provides long-term follow-up data up to 21 years post-anthracycline chemotherapy completion using a comprehensive dataset. The aim was to establish the incidence of late onset cardiovascular toxicity, thus informing long-term cardiotoxicity surveillance protocols to enable the early recognition and treatment of anthracycline-induced cardiotoxicity.

The cardiotoxicity incidence within this adult breast cancer pharmacogenetics cohort was calculated as 35.8%, with 29 out of 81 patients who had received an echocardiogram since chemotherapy completion diagnosed with anthracycline-induced cardiotoxicity. With the median duration of patient follow-up since chemotherapy completion 18 years, and all patients followed-up for at least 10 years. This study therefore represents one of the longest follow-up durations for the evaluation of cardiotoxicity in adult patients with breast cancer (Zambetti et al., 2001, Shulman et al., 2014). Furthermore, the anthracycline-induced cardiotoxicity incidence within the analysis (35.8%) was much greater compared to other breast cancer cohort studies, with their incidence ranging from 1.9 to 11.4% (Buzdar et al., 1985, Jensen et al., 2002, Swain et al., 2003, Ryberg et al., 2008). The extended follow-up duration of this study could account for the increased anthracycline-induced cardiotoxicity incidence, providing more time for the cardiotoxicity phenotype to develop. These findings are of considerable clinical significance considering the data from Cardinale et al. (2015), who reported 98% of cardiotoxicity cases (n=221) presenting within the first year since chemotherapy completion. These data indicates that cardiotoxicity continues to emerge even after the first-year post-chemotherapy completion, with 21 cardiotoxicity patients (72.4%) diagnosed beyond this period in the cohort analysed in this study. This is supported by a median duration to diagnosis calculated at 8.3 years, with cardiotoxicity cases even detectable up to 19 years post-chemotherapy completion. It is acknowledged that a limitation of the study is that patients were not subjected to regular echocardiograms during

follow-up, with echocardiograms only completed for patients presenting with symptoms or other cardiovascular-related reasons. Therefore, it is important to be aware that as most patients had not received cardiac imaging within their first year following chemotherapy, the precise onset of cardiotoxicity was unknown, that is until first cardiac imaging was performed. This strengthens the argument for screening patients for cardiotoxicity, especially as these observations indicate that until evidence-based heart failure treatment is initiated in patients with anthracycline-induced cardiotoxicity, cardiac function remains impaired and patients are at high risk of progression towards symptomatic heart failure.

These findings provide valuable insight for cardiotoxicity surveillance protocols, advocating that anthracycline-induced cardiotoxicity monitoring should continue beyond 12-24 months post-treatment, which currently only receives level III B evidence recommendations within adult cardio-oncology guidelines (Curigliano et al., 2020, Stone et al., 2021). Moreover, this encourages the development and application of cardiotoxicity risk assessment tools, to provide targeted screening for those at greatest risk of cardiotoxicity and determine which patients required long-term cardiology follow-up. To enhance this study, regular echo monitoring for study participants (i.e. annually or biannually), would not only enable the development of cardiotoxicity to be established earlier, promoting earlier treatment intervention, but also provide confidence intervals for the time of cardiotoxicity diagnosis. Based upon these findings, it is advocated that at the present time, asymptomatic cardiotoxicity screening should not be discontinued, even in patients beyond 2 years post-anthracycline chemotherapy. Cost-effectiveness research in childhood cancer survivors also supports ongoing echocardiography assessment for anthracycline-induced cardiotoxicity. Health economic analyses have proposed a risk-based approach to surveillance or an incremental reduction in screening intervals should be incorporated into clinical guidelines, thus ensuring a clinically effective strategy towards late-onset anthracycline-induced cardiotoxicity (Yeh et al., 2014, Ehrhardt et al., 2020).

The thresholds considered diagnostic of cardiotoxicity may provide a source of variability when reporting anthracycline-induced cardiotoxicity incidence. For identifying cases of anthracycline-induced cardiotoxicity, a definition of a reduction in LVEF to <55% on echo, and a greater than 10% reduction in LVEF from baseline was defined. Cardiotoxicity definitions have become a subject of much debate, with significant heterogeneity existing across the

literature regarding the optimal diagnostic threshold (Lambert J, 2016, Chung et al., 2018). Fractional shortening (FS), used principally as a measure to detect cardiotoxicity in paediatric and adolescent patients, is similarly exposed to the debate regarding correct diagnostic thresholds (Von Hoff et al., 1977, Plana et al., 2014). The new British-Society of Echocardiography (BSE) Cardio-Oncology Guidelines recognise an LVEF <50% as significant for chemotherapy-related cardiotoxicity, whilst a LVEF between 50-54% is considered 'borderline' (Dobson et al., 2021). Echocardiography is recognised as having inter-observer variability, therefore adopting a 'borderline' threshold theoretically prevents initiation of life-long treatment in patients with a falsely positive scan indicating cardiotoxicity. Despite subclinical cardiotoxicity considered as the asymptomatic precursor to congestive cardiac failure, randomised controlled trial evidence has shown reversibility of the diagnosis if detected early and with prompt initiation of ACEi therapy (Cardinale et al., 2015). Although the reported meta-analysis incidence of 13.8% in adult patients, there remains a paucity of prospective clinical data regarding the implications of a 'borderline' subclinical cardiotoxicity (Jang et al., 2019).

In the preclinical cellular analysis stages of this study, anthracyclines were shown to induce cardiomyocyte damage, with the remaining functional cardiomyocytes appearing to compensate for the incurred cardiomyocyte losses. Histological analysis also demonstrated enhanced fibrotic responses to maintain overall myocardial structural integrity and attempt to preserve cardiac function. It is therefore argued that any reduction in cardiomyocyte numbers caused by anthracycline exposure renders patients susceptible to myocardial dysfunction, especially as overall cardiomyocyte numbers continue to decrease with ageing and additional myocardial injury or stress. Extrapolating these laboratory-based findings into clinical practice, a reduction in LVEF to below the 'normal' range (i.e. <55%) is classed as clinically significant, necessitating initiation of evidence-based cardiotoxicity treatment strategies. Broadening of the anthracycline-induced cardiotoxicity diagnosis therefore incorporated additional cardiotoxicity cases, with 5 patients identified within the 50-54% borderline classification. However, when applying the BSE <50% threshold, anthracycline-induced cardiotoxicity incidence was still 29.6% and therefore remained substantially higher than comparable cardiotoxicity studies.

Evaluation of therapy and patient-related anthracycline-induced cardiotoxicity risk factors within the adult breast cancer patient cohort, failed to identify factors associated with the development of cardiotoxicity. Although calculated hazard ratios indicated a positive trend towards increased age at treatment completion, increased cumulative anthracycline dose, mediastinal radiotherapy and female sex, differences were not statistically significant as indicated by the confidence intervals (**Table 5.2**). Although this lack of statistical significance may reflect the relatively small sample size (n=81), these findings support the hypothesis of a multi-factorial cause for anthracycline-induced cardiotoxicity, with potential unidentified risk factors including genetic polymorphisms responsible for the cardiotoxicity phenotype (Armstrong et al., 2013). Larger scale prospective studies are therefore required to strengthen the evidence for incorporating cardiotoxicity risk factors with anthracycline-induced cardiotoxicity prediction models.

Interestingly, the sub-analysis evaluating the association between baseline blood pressure and anthracycline-induced cardiotoxicity demonstrated a statistically significant relationship (**Figure 5.2**). Acknowledging the low patient numbers within this study, a higher baseline diastolic blood pressure was seen with the cardiotoxicity group (85.2 ± 6.0 mmHg) compared to the patients without cardiotoxicity (74.3 ± 5.4 mmHg)(p=0.005). Although this elevation in diastolic blood pressure remains below the level considered for diastolic hypertension (≥90mmHg), it is still considered within the 'pre-hypertension' classification (80-90mmHg), which is strongly associated with the development of hypertension and higher cardiovascular risk (Williams et al., 2018, Flint et al., 2019).

Patients with a high-normal blood pressure or 'pre-hypertension', defined by the ESC arterial hypertension guidelines as blood pressure in the range 130-139/85-89mmHg, are considered at risk of cardiovascular events such myocardial infarction, stroke and heart failure (Williams et al., 2018), with almost two-thirds of patients developing hypertension within 24 months (Julius et al., 2006, van der Merwe, 2017). Endogenous physiological changes involving excessive RAS stimulation, nitric oxide dysfunction, reactive oxygen species synthesis, inflammatory cytokines, and impaired baroreceptor control are implicated for progression to established hypertension (Albarwani et al., 2014). Increases in cardiac preload, afterload and myocardial wall stress caused by elevated blood pressures renders the heart vulnerable to progressive ventricular failure. Further cardiomyocyte damage caused by exposure to

anthracycline chemotherapy, overwhelms cardiac compensatory mechanisms, leading to the development of clinical heart failure. Therefore, early identification and treatment of hypertension is essential to prevent both left ventricular dysfunction and interruptions to cancer chemotherapy and premature treatment discontinuation (Szmit et al., 2014).

Patients within the cardiotoxicity group (**Figure 5.2**) also demonstrated a higher mean blood pressure compared to patients without cardiotoxicity (138.2mmHg vs. 131.4mmHg). Although this study included low patient numbers and these results were not statistically significant, the mean blood pressure values fall into the 'pre-hypertensive' range. Hypertension has been reported as the most common comorbidity (38%) encountered in patients diagnosed with malignancy (Fowler et al., 2020). Until now, the role of baseline blood pressure as a predictor and contributor for the development of anthracycline-induced cardiotoxicity has perhaps been under-recognised, with an association reported only within isolated clinical studies (Hershman et al., 2008, Szmit et al., 2014). A recent systematic review evaluating the clinical significance of hypertension in patients treated with anthracycline chemotherapy, confirmed that elevations in baseline blood pressure increase adult patients' susceptibility to anthracycline-induced cardiotoxicity (Philip et al., 2022). Furthermore, recent evidence has strongly supported the measurement and treatment of elevated diastolic blood pressure, which independently predicts risk of adverse cardiovascular outcomes (Flint et al., 2019). The small retrospective analysis (**Figure 5.2**) (**Table 5.3**) demonstrated a positive correlation between diastolic blood pressure and the development of cardiotoxicity. Whilst prospective studies, with greater patient numbers are requirement to confirm these findings, these results indicate the potential role for blood pressure monitoring prior to anthracycline chemotherapy, in addition to observing for elevations during and soon after treatment. Evidence-based clinical guidelines are yet to advocate prescribing anti-hypertensive treatment in patients within the 'pre-hypertensive' range unless additional cardiovascular risk factors (i.e. diabetes, hypercholesterolaemia), however, there may be a role for initiating cardioprotective therapy (e.g. ACE inhibitors or ARBs) even at this stage, given this new found association with cardiotoxicity and recognising anthracycline chemotherapy amongst these cardiovascular risk factors (Williams et al., 2018, Boffa et al., 2019, Philip et al., 2022).

Given the strong influence of blood pressure upon cardiovascular risk, the QRISK3® cardiovascular risk scores of patients diagnosed with cardiotoxicity (n=29) and patients

without cardiotoxicity (n=52) were compared. This represents a patient's percentage risk of developing a heart attack or stroke over the next 10-years. Within the cardiotoxicity cohort, the mean 10-year cardiovascular risk score was 20.2% (s.d.±11.1), whilst the mean score in the non-cardiotoxicity cohort was 16.2% (s.d.±11.5). Although this difference was not statistically significant (p=0.099), there is a trend showing that patients with a higher cardiovascular risk score are more likely to develop anthracycline-induced cardiotoxicity. This represents a rather timely analysis following the release of the European Society of Cardiology 2020 guidelines for baseline cardiovascular risk assessment in cancer patients (Lyon et al., 2020). The introduction of baseline cardiovascular risk assessment prior to receiving oncology therapies aims to stratify patients according to their risk of developing cardiovascular complications either during or after cancer treatment. In addition to cardiac imaging and biomarker analysis, oncologists are encouraged to screen for cardiovascular risk factors to identify patients at an increased risk of cardiovascular complications. Patients recognised as high risk should be offered a personalised approach to their care, seeking to eliminate adverse cardiovascular outcomes with appropriate cardiology guidance, whilst also ensuring safe completion of their chemotherapy (Lyon et al., 2020).

Successfully applying a personalised approach to patient care can reduce the cardiovascular toxicities of cancer therapies, whilst cardiovascular mortality in patients with cancer can be substantially reduced by active risk factor modification (Lyon et al., 2020). Beyond lifestyle modifications such as smoking cessation, encouraging a healthy diet, maintaining a healthy weight and exercising, all patients with established cardiovascular disease should be offered evidence-based secondary prevention medication as recommended in NICE guidance [CG181]. In addition to lifestyle advice, patients should have their underlying cardiovascular risk assessed using a well-validated risk evaluation tool such as QRISK, JBS3, Framingham or ACC/AHA. NICE recommends using QRISK®3 (https://qrisk.org/three) which is based on outcomes from over 2.3 million GP records in the UK. Patients with a calculated 10-year risk of at least 10% should be offered lipid lowering treatment with atorvastatin 20mg daily, which is highly cost effective (under 3p per tablet) and very well-tolerated (Jones et al., 2007, Duerden et al., 2015).

Breast cancer is the most common cancer in the UK and shares multiple risk factors with cardiovascular disease including age, diet, smoking, obesity, alcohol, physical activity and use

of hormonal therapies (Mehta et al., 2018). Breast cancer survivors are therefore recognised as having a greater risk of cardiac disease and cardiovascular mortality compared with the general population (Park et al., 2017, Ramin et al., 2020). In the study of the cardiovascular risk profiles of patients treated for cancer at Newcastle upon Tyne Hospitals NHS Foundation Trust, an unmet need was strongly emphasised regarding primary cardiovascular risk prevention, as 67% of patients were identified as either 'high' cardiovascular risk (i.e. 10-year cardiovascular risk score >20%) or 'medium' cardiovascular risk (i.e. 10-20%). However, despite this high cardiovascular risk burden, only 51% of these patients were receiving effective primary prevention therapy as recommended by NICE guidelines. These results were not specific to breast cancer, with patients from lung and prostate cancer follow-up clinics demonstrating the same trends.

Despite significant improvements to cancer therapy, 5-year age-standardised lung cancer survival in England and Wales has only increased by 3% over the past 40 years, with lung cancer patients also experiencing a high burden of cardiovascular morbidity and mortality (Quaresma et al., 2015). Cardiovascular co-morbidity in the lung cancer population has been shown to have a significant impact upon short and long-term survival, with recent literature indicating the true burden of cardiovascular disease within this high-risk patient population could even be underestimated (Bryant et al., 2010, Rodríguez et al., 2013, Speirs et al., 2017, Johnson-Hart et al., 2018). The relationship between lung cancer and cardiovascular disease is perhaps unsurprising given the shared risk factors such as ageing, smoking, obesity, and poor diet, in addition to cardiac exposure from chest radiotherapy all accelerating coronary artery atherosclerosis (Kravchenko et al., 2015, Madan et al., 2015, Speirs et al., 2017). With this, over 80% of patients with lung cancer were identified as having either a high or medium 10-year cardiovascular risk. Despite having a cardiovascular risk score over 10% and being highly amenable to cardio-protective therapy, only 53% of these 'at risk' patients were prescribed the recommended cardiovascular prevention therapy.

The cardiovascular risk profile of patients diagnosed with prostate cancer is similar to lung cancer, with a high prevalence of cardiovascular risk factors including smoking, obesity, male sex, increasing age and physical inactivity identified (Leong et al., 2020, Pinthus et al., 2020). The use of androgen deprivation therapies, including gonadotrophic releasing hormone for prostate cancer has also been shown to significantly increase the risk of cardiovascular events

(Gandaglia et al., 2014, Bhatia et al., 2016). In this analysis of patients with prostate cancer attending the clinic, all patients classified as 'high' or 'medium' risk based upon their calculated 10-year cardiovascular risk score, only 53% of these patients prescribed cholesterol lowering primary prevention therapy. These results are supported by a retrospective analysis from Canada of 100 consecutive men with localised prostate cancer at a mean age of 73 years. This study from Davies et al. (2015) found that 25% had pre-existing vascular disease and an additional 74% had risk factors placing them in the high or intermediate Framingham risk categories, with only 26% receiving primary prevention treatment with statins at the time of assessment (Davis et al., 2015). These results are of strong clinical significance as meta-analysis data has confirmed statin treatment to reduce both prostate-specific and all-cause mortality by 24% (Raval et al., 2016). This study therefore provides the necessary platform to develop recommendations for active cardiovascular risk evaluation in patients with cancer, and to stimulate large-scale prospective studies to support baseline cardiovascular risk assessment in cancer patients receiving cardiotoxic chemotherapies.

The combined data from these three studies supports recent large-scale cohort and meta-analyses data emphasising the need for cardiovascular risk screening and prevention in patients with cancer. Clinical evidence has shown cardiovascular mortality in patients with cancer can be reduced through active risk factor modification and early identification of cardiovascular toxicities (Zhong et al., 2015, Yuan and Li, 2018, Strongman et al., 2019). Although risk prediction models specific to cardiotoxicity are in development, there is the potential for utilising these models within prospective clinical trials. For example, researchers could consider randomising patients to either a cardiovascular risk modification approach or standard care, to evaluate the influences of these risk-based strategies upon prognosis and cardiotoxicity outcomes (Abdel-Qadir et al., 2019). Successful application of these approaches would further support the hypothesis for pro-active risk factor modification to reduce cardiovascular mortality. This provides confidence for initiating this highly cost-effective strategy for reducing cardiovascular events in patients with cancer and promote long-term cancer survival. Furthermore, recent evidence has even found that statin administration in patients receiving anthracycline chemotherapy may reduce the risk of anthracycline-induced cardiotoxicity and enhance cardiovascular outcomes (Obasi et al., 2021, Abdel-Qadir et al., 2021).

The widespread use of anthracycline chemotherapy within adult and paediatric oncology has contributed to the significant improvements in cancer survival rates. Cancer survivorship studies have demonstrated that long-term cancer survivors are exposed to cardiotoxic effects induced by anthracycline chemotherapy. Despite significant advances in heart failure care and pharmacotherapy, paediatric hospitalisation and mortality rates from cardiomyopathy remain high (Arola et al., 1997, Tsirka et al., 2004, Towbin et al., 2006, Andrews et al., 2008, Rossano et al., 2012). Evidence taken from adult patients has shown that advanced, symptomatic anthracycline-induced cardiomyopathy responds poorly to treatment, with mortality as high as 50%, with a median survival of 162 days from symptom onset (Nielsen et al., 1990, Ryberg et al., 1998). Furthermore, clinical research has demonstrated that clinical symptoms of heart failure correlate poorly with left ventricular function. These findings are highly significant given the poor prognosis of congestive cardiac failure in paediatric patients, with delays in recognising cardiac dysfunction and initiating heart failure treatment associated with even greater increases in patient mortality (Lipshultz et al., 1991, Guglin et al., 2012). Since appreciating the clinical and prognostic value of early heart failure diagnosis, monitoring patients with echo and advanced cardiac imaging has improved cardiotoxicity outcomes. The estimated prevalence of subclinical cardiotoxicity in paediatric patients treated with anthracyclines is recorded as substantially higher (56%) than in adult patients (13.8%) (Lipshultz et al., 1991, Kremer et al., 2002, Jang et al., 2019). These alarming statistics therefore represent a substantial number of patients who would likely benefit from early cardiotoxicity treatment.

The clinical literature has also provided important knowledge that a causal relationship exists between cumulative anthracycline dose and cardiac events, which has helped to identify patients at risk of cardiotoxicity and modify treatment protocols (Nysom et al., 1998, van Dalen et al., 2006, Temming et al., 2011). These studies have informed international recommendations which advocate increased cardiac monitoring for subclinical cardiotoxicity, especially patients with cumulative doses above 250mg/m^2. However, the same retrospective cohort studies have established that even patients receiving low anthracycline doses (<250mg/m^2) can develop cardiotoxicity, making cardiotoxicity risk prediction challenging (Sorensen et al., 2003, Armenian et al., 2015). *(The incidence of CHF is <5% with*

cumulative anthracyclines exposure of <250 mg/m^2; approaches 10% at doses between 250 and 600 mg/m^2; and exceeds 30% for doses >600 mg/m^2 (Armenian et al., 2015)).

A further prospective study by the Cardinale group assessed cardiac function within adult patients with breast cancer during and after anthracycline chemotherapy, in a follow-up period of 4 years. As previously discussed, the results of this study revealed that 98% of adult cardiotoxicity cases occurred within the first year, with the median time from anthracycline chemotherapy completion 3.5 months. The results from the study herein demonstrate that in long-term paediatric cancer survivors, cases of anthracycline-induced cardiotoxicity continue to be diagnosed up to 20 years after the completion of chemotherapy, with a median of 4.1 years. Of the 41 (23.7%) patients diagnosed with anthracycline-induced cardiotoxicity, 48.8% were diagnosed with cardiotoxicity more than 5 years after chemotherapy completion.

A systemic review, which included 25 studies of childhood cancer survivors, demonstrated the variable incidence of cardiotoxicity, with subclinical cardiotoxicity ranging from 0 to 57%. One reason for these reported differences was the definition of cardiotoxicity, with FS diagnostic thresholds interpreted as either <28%, <29%, or <30% (Kremer et al., 2002). Based upon paediatric echo data which considers the normal values for FS in infants and children to be between 28 and 46%, 28% was considered as the lower limit of normal within this study to establish a cardiotoxicity diagnosis (Tissot et al., 2018). Therefore, the cardiotoxicity incidence within this study was 23.7% (41 patients). If a FS threshold of <29% was applied to the data in this study, the cardiotoxicity incidence would inevitably increase to 32.9% (57 patients), with a considerable increase to 41.0% (71 patients) if the <30% threshold was used. Whilst these revised statistics are comparable to the anthracycline-induced cardiotoxicity incidence within other paediatric cohort studies included with the analysis, another reason for the incidence variability is due to the length of follow-up utilised. Collecting long-term follow-up data in young patients is challenging, especially within the context of globalisation and increasing population mobility. In this context, one strength of the data analysed in this study is that it was collected from clinics in the North East of England, which according to recent Office of National Statistics data (ONS), has consistently shown the lowest migration outflow within the United Kingdom over the last 10 years (Park, 2021). This was consequently reflected within the young cancer survivor population analysed in the study, creating stability within long-term follow-up clinics, with patients frequently returning for appointments at

their initial cancer treatment hospital despite moving out of the region. This is maximised as an advantage by including patients followed-up for more than 5 years, with the median follow-up post-anthracycline chemotherapy 9.3 years, and the maximum follow-up 26.3 years. Whilst the median time post-chemotherapy to anthracycline-induced cardiotoxicity diagnosis was 4.1 years, 11.6% of all patients within this study were diagnosed with cardiotoxicity beyond 5 years following chemotherapy completion, equating to almost half (48.8%) of patients diagnosed with cardiotoxicity. These data are strongly influenced by 34.1% of cardiotoxicity patients diagnosed either during anthracycline chemotherapy or within the first-year post-treatment. Evaluating other long-term paediatric cardiotoxicity studies, van der Pal et al. (2010) demonstrated a similar anthracycline-induced cardiotoxicity incidence, finding 27% of young adult childhood cancer survivors 5 years or more post-anthracycline chemotherapy developed cardiotoxicity. The FS threshold for this study was <30%, therefore if the cohort was evaluated with this threshold, the anthracycline-induced cardiotoxicity incidence would be 41%.

Steinherz et al. (1991) also evaluated the anthracycline-induced cardiotoxicity incidence within long-term survivors of paediatric malignancies with a comparable follow-up of between 4 to 20 years. The overall anthracycline-induced cardiotoxicity incidence of 23% within this study was similar to the outcomes of the study herein, however they excluded patients with a doxorubicin equivalent dose of less than 200mg/m^2, and adopted a higher FS threshold of < 29% (Steinherz et al., 1991). Subsequent publications have established that there is no safe anthracycline dose, with cardiomyopathy seen even when low cumulative doses have been administered (Sorensen et al., 1997, Lipshultz et al., 2005). Applying the same dosing threshold upon the paediatric cancer survivor patient cohort, and only including patients receiving >200mg/m^2 doxorubicin, would have excluded almost 25% of cardiotoxicity cases. Therefore, whilst risk stratification and echo surveillance strategies may be informed by cumulative anthracycline doses, the data in this study advocates that all patients should be considered for long-term cardiotoxicity monitoring. Within adult international guidelines, monitoring strategies during therapy are well defined, however, specifying the frequency of monitoring post-anthracycline chemotherapy appears more complex (Čelutkienė et al., 2020, Curigliano et al., 2020). As discussed, early detection of cardiotoxicity is imperative to improved clinical outcomes, however, 5-yearly cardiac imaging intervals may find patients

developing cardiotoxicity between scans and risk several years of untreated left ventricular dysfunction which has the potential to progress rapidly to symptomatic cardiac failure.

Retrospective cardiomyopathy risk stratification of childhood cancer survivors identified with the cardiotoxicity phenotype (**Table 5.5**) demonstrated that 63.4% of patients were categorised as high risk, based upon cumulative anthracycline dose and chest radiotherapy dose. According to the recommendations, these patients should be screened at least 5-yearly and potentially more frequently for cardiomyopathy. Of the remaining cardiotoxicity patients, 34.1% were identified as moderate risk, whilst 2.4% were considered as low risk. According to the International Harmonization Group Recommendations, patients categorised as either moderate or low risk of cardiomyopathy are advised to receive clinical and imaging surveillance every 5 years (Ehrhardt et al., 2020).

Wang et al. (2001) completed a review of the clinical course of asymptomatic left ventricular dysfunction, with a combination of 5 randomised, controlled trials revealing the incidence of congestive cardiac failure (CCF) to be between 4.9% and 20%, with annual average mortality rates between 5.1% and 10.5% (Wang et al., 2003). Whilst in a long-term retrospective cohort study specific to paediatric anthracycline-induced cardiotoxicity, the cumulative incidence of CCF was found to be 2.8%, with the estimated risk increasing to 3.3% after 10 years, and 4.8% after 15 years post-anthracycline chemotherapy, respectively (Kremer et al., 2001). Whilst there is the agreed consensus opinion regarding cardiomyopathy surveillance post-anthracycline chemotherapy, further prospective studies are required to validate these risk prediction strategies and provide cost effective screening for anthracycline-induced cardiotoxicity.

The pharmacological analysis within the anthracycline-induced cardiotoxicity cohort demonstrated that 39.5% of patients were receiving heart failure therapy, with ACEi the most frequently prescribed therapy (36.8%), followed by beta-blocker therapy (13.2%). These statistics demonstrate a potential under-treatment of anthracycline-induced cardiotoxicity within the paediatric long-term follow-up clinic. One explanation for this is potentially the different FS threshold (<28%) which was applied compared with other long-term paediatric cardiotoxicity studies, with the selection of this lower limit of normal justified based upon the available meta-analysis and echo data. A further explanation related to the ambiguity around treatment for subclinical anthracycline-induced cardiotoxicity in paediatric patients.

Paediatric clinical guidelines advocate the use of ACEi for treatment of anthracycline-induced cardiotoxicity, with randomized controlled trial evidence supporting their positive cardiac effects combined with a favourable short-term safety profile in patients treated for hypertension or heart failure. Patients should be closely monitored for adverse events relating to ACEi, including renal impairment, hypotension and hyperkalaemia (Momma, 2006, Snauwaert et al., 2017, van der Meulen et al., 2018). However, there remains a lack of long-term safety data for ACEi, especially relating to their influence upon paediatric growth, development and maturation. Furthermore, uncertainty still exists around their long-term benefits for preventing progressive cardiomyopathy (Sieswerda et al., 2012, Kirk et al., 2014). A randomised controlled trial evaluating the effectiveness of ACE inhibition compared to placebo in paediatric cancer survivors with asymptomatic anthracycline-induced cardiomyopathy failed to show any statistical difference between the clinical trial end points, with an increased reporting of side effects within the ACEi group (Silber et al., 2004). Whilst a retrospective study of doxorubicin-treated survivors of childhood cancer demonstrated early cardiovascular improvements with ACEi therapy, before a deterioration in left ventricular structure (i.e. progressive wall thinning) and function (i.e. increased afterload, fall in FS and contractility) after 6 years of therapy, suggesting that clinical benefits may only be transient (Lipshultz et al., 2002). Although early ACEi treatment may prevent late cardiotoxicity and reduce cardiac biomarker levels (i.e. troponin, proBNP and CK-MB), there is further need for prospective studies evaluating the cardiovascular protective effects of ACEi in paediatric cancer survivors (Gupta et al., 2018, Lyon et al., 2022).

The differences between adult and paediatric patient responses to ACEi may be best explained by the stages of cardiomyocyte maturation, the composition of supporting cells (e.g. fibroblasts, endothelial cells and cardiac progenitor cells) and the cardiomyocyte response to anthracycline-induced injury (Olivetti et al., 1991, De Angelis et al., 2016). Through histological analysis, anthracycline chemotherapy is known to induce loss of cardiomyocyte mass within cardiac tissue (Jordan et al., 2018, Neilan et al., 2012). Although cardiomyocytes have demonstrated some mild regenerative capacity, human cardiomyocytes are post-mitotic, meaning that in conditions causing significant myocyte cell death, the loss of cardiomyocytes is largely irreversible. Cardiomyocytes do, however, undergo hypertrophic growth responses, with functional myocytes adequately compensating for any myocyte losses

incurred to maintain cardiac output. Whilst these myocyte losses initially impair myocardial contractility and the growth potential of the myocardium, cardiomyocytes have been observed to undergo hypertrophic growth responses, with functional myocytes adequately compensating for any myocyte losses incurred to maintain cardiac output, up to a threshold limit. The gradual loss of cardiomyocytes experienced beyond adolescence, places added functional strain upon the remaining myocytes, diminishing the cardiac contractile reserve and the capacity to cope with physiological stressors. Unless treated, these alterations in cardiac haemodynamics can result in long-term compensatory effects, such as fibrosis and reduced myocardial contractility, thus accelerating cardiac functional decline and the presentation of symptomatic heart failure (Olivetti et al., 1991, De Angelis et al., 2016, Lipshultz et al., 2014). This progressive myocyte loss is not unique to children, with adult patients also demonstrating a reduction in left ventricular mass post-anthracycline chemotherapy. Whilst cardiomyocyte hypertrophy and replacement fibrosis can improve extracellular volume, age-related cardiomyocyte degradation limits the contractile potential of the heart and leads to cardiac failure (Ferreira de Souza et al., 2018, Jordan et al., 2018). There is however a threshold of both insult and cellular loss that can be accommodated, beyond which the heart can no longer adapt and maintain full function. At this point, cardiac stresses mount and compensatory processes become inadequate. Subsequent pathological cardiac remodelling including left ventricular wall thinning and dilatation, leads to myocardial dysfunction and clinical heart failure (Mercurio et al., 2016). Crucially, this adverse pathological sequence is dependent upon the timing of anthracycline-induced cardiotoxicity diagnosis, with early diagnosis and intervention shown to be the greatest determinants (Kamphuis et al., 2020).

Overall, both paediatric and adult cancer survivors exposed to anthracycline chemotherapy are a risk of developing late-onset anthracycline-induced cardiotoxicity. The incidence of anthracycline-induced cardiotoxicity within the long-term paediatric cancer survivor cohort was 23.7%, whilst within the adult cancer survivor cohort the incidence was 35.8%, with patients developing cardiotoxicity up to 20 years post-anthracycline chemotherapy completion. Accordingly, continuation of long-term surveillance for anthracycline-induced cardiotoxicity is advocated, with early treatment intervention for detected cases of anthracycline-induced cardiotoxicity. Further prospective clinical studies are still required to

establish the exact length of cardiotoxicity treatment and preventative measures, with the combined role of cardiac biomarkers offering a pragmatic solution to manage resources.

The results from this chapter demonstrate that patients with cancer have an increased risk of cardiovascular disease, either as a long-term consequence of cancer therapies or high prevalence of cardiovascular risk factors. This is supported by large-scale cohort data, with Stolzfus et al. (2020) observing in a study of 7.5 million patients with cancer, the rate of cardiovascular disease-related mortality was more than double (2.24 times) compared to the general population, and increasing during long-term follow-up (Stoltzfus et al., 2020). Similarly, Strongman et al. (2019) identified that the risks of cardiovascular disease (heart failure, coronary artery disease and arrhythmias) were elevated in cancer survivors when compared with matched controls, and maintained even up to five years after diagnosis (Strongman et al., 2019). Less well explored however, is whether a relationship exists between cancer and cardiovascular disease beyond cancer therapies and shared risk factors.

Parallels between inflammatory responses detected in cardiovascular disease and those observed in malignancy have been investigated. Systemic low-grade, chronic inflammation has been shown to promote both coronary artery atherosclerosis and chronic heart failure, with similar immunological pathways identified in tumour initiation and development (Mantovani et al., 2008, Adamo et al., 2020). Evidence of these shared mechanisms was presented in the CANTOS trial, which found that administering anti-inflammatory therapy (Canakinumab, a cytokine-based therapy targeting interleukin-1) to patients with established cardiovascular disease and elevated high-sensitive C-reactive protein (hsCRP) levels, not only lowered the incidence of future cardiovascular events, but also reduced the incidence of lung cancer and associated mortality (Ridker et al., 2017a, Ridker et al., 2017b). Therapeutic blockade of the interleukin-1 pathway therefore demonstrates promise in preventing cardiovascular events, whilst interleukin-1 activation has been shown separately to promote cancer initiation and progression (Szekely and Arbel, 2018, Gelfo et al., 2020). This relationship is further recognised by raised plasma hsCRP levels associated with both recurrent cardiovascular events and the development of cancer, with this biomarker of low-grade inflammation potentially predictive of cancer progression within specific tumour types (Van't Klooster et al., 2019, Hart et al., 2020). Together, these findings indicate that even prior to receiving cancer therapeutics, patients may have an increased susceptibility to developing

cardiovascular disease, with future clinical trials necessary to support inflammatory pathway inhibition for the prevention of cancer and major cardiovascular events.

Finally, the vast majority of research to date into breast cancer and cardiovascular risk has been principally concerned with the direct effects of treatment and risk factors causing cardiovascular events within these patients, rather than the converse. It therefore remained to be determined whether increased cardiovascular risk influences breast cancer recurrence This was appraised through analysis of patient 10-year cardiovascular risk estimates, as calculated by QRISK3® (**Table 5.6**). Preclinical studies had indicated increases in breast cancer growth following myocardial infarction (MI), which seemingly induces an immunosuppressive state promoting unregulated tumour growth (Koelwyn et al., 2020). These findings have even been translated into the clinic, with retrospective patient studies demonstrating an increased cancer incidence associated with thromboembolic events in patients with undiagnosed cancer (Hasin et al., 2016, Koelwyn et al., 2020).

Through analysis of cardiovascular risk scores, no association between cardiovascular risk estimates of patients diagnosed with breast cancer recurrence and patients who remained cancer free following completion of anthracycline-based chemotherapy was identified. These results therefore suggest that the presence of known cardiovascular risk factors alone may be insufficient to promote breast cancer recurrence. This lack of association could be explained by the need for a certain level of systemic inflammatory response, required to trigger a sufficient immune response and promote cancer regrowth. A major cardiac event, for example a myocardial infarction or symptomatic heart failure may be a necessary stimulus. Moreover, no association was demonstrated after analysing the relationship between cardiotoxicity and breast cancer recurrence. This suggests that cardiotoxicity, unless highly symptomatic, maybe an insufficient physiological stressor for creating an immune response promoting cancer growth. Interestingly, heart failure registries indicate an association with cancer incidence, which is perhaps greater than the impact of the individual risk factors (Banke et al., 2016, Koene et al., 2016). Although patient sample sizes were small, this analysis does provide further stimulus for research into 'reverse cardio-oncology', and towards understanding the complex interaction between cardiovascular co-morbidities and cancer progression.

5.3.1 Conclusions

Cardio-oncology has emerged from the success of modern haematology and oncology treatments, with improved cancer survival rates meaning these patients are susceptible to developing cardiovascular disease over the long-term. Evidence from this chapter emphasises the importance of baseline and ongoing cardiovascular risk assessment in patients undergoing chemo-radiotherapy, with a high burden of cardiovascular risk factors and cardiovascular disease identified within three cancer patient cohorts. Early identification and personalised management of these risk factors, not only helps to reduce the incidence of cardiovascular disease, but also ensures patients continue to receive their evidence-based cancer therapies.

Anthracyclines are associated with cardiotoxicity, which may by diagnosed either during, or after patients have received their cancer treatment. Analyses from both the adult and paediatric studies provides strong evidence of patients developing left ventricular dysfunction even several years after completing their cancer chemotherapy. This strengthens the argument for long-term cardiotoxicity surveillance, to support both early diagnosis and timely management of these complications attributed to previous cancer treatments. To optimise these pathways of care and enhance long-term cancer survivorship, risk stratification is strongly advocated to ensure patients at high risk of developing cardiotoxicity are easily identified, receive regular monitoring, and are started on vital cardioprotective treatments. Finally, these studies provide the stimulus for further large-scale clinical trials evaluating long-term cardiotoxicity surveillance, informing how to best allocate cardio-oncology resources, and administer evidence-based cardioprotective treatments to improve overall cancer outcomes.

Chapter 6. General Discussion

Anthracycline-induced cardiotoxicity threatens to limit the recent improvements in long-term cancer survival, with patients exposed to anthracycline chemotherapy at increased risk of developing cardiovascular disease, in both the acute and chronic settings. Given the significant morbidity and mortality associated with anthracycline-induced cardiac dysfunction, early detection of cardiotoxicity has important therapeutic and prognostic implications (Felker et al., 2000, Wojtacki et al., 2000, Cardinale et al., 2010). The principal aim is to ensure patients receive the maximum therapeutic benefit from their evidence-based cancer therapies, whilst limiting the off-target toxicities associated with treatment. The field of cardio-oncology continues to develop early preventative strategies, aiming to optimise the management of cardiovascular disease for patients with cancer, and by promoting a multi-disciplinary patient-centred approach.

Despite notable advancements within cardio-oncology, there remains an incomplete understanding of the mechanisms, the pathophysiology, and the optimal treatment strategies specific to anthracycline-induced cardiotoxicity. Pre-clinical and clinical studies have demonstrated that RAAS inhibitors can reduce and even prevent anthracycline-induced cardiotoxicity (van Dalen et al., 2011, Kalam and Marwick, 2013, Cardinale et al., 2015, Conway et al., 2015). The efficacy of ACEi and ARBs for the treatment of other cardiovascular disorders (i.e. hypertension, myocardial infarction, heart failure) is mainly through their optimisation of cardiac haemodynamics and associated with improvements in cardiac ejection fraction. However, their mechanisms specific to the treatment and prevention of anthracycline-induced cardiotoxicity are unknown, whilst their optimal timing and dosage in this setting is still being investigated.

6.1 Role of angiotensin signalling in anthracycline-induced cardiotoxicity

The primary aim of this thesis was to elucidate the precise mechanisms for why ACEi exhibit cardioprotective effects for the prevention of anthracycline-induced cardiotoxicity, and explore neurohormonal activation in response to myocardial injury. Through analysis of the angiotensin II receptor, the intention was to evaluate the relationship between the angiotensin signalling pathway and anthracycline-mediated cardiotoxicity. Furthermore, given the strong clinical incentives for preventing cardiotoxicity, the value of researching

genetic predisposition and cardiotoxicity biomarkers associated with late-onset cardiotoxicity was recognised, to support accurate cardiotoxicity risk prediction and enhance long-term cardio-oncology surveillance strategies.

To achieve these aims, protocols for AC10 cardiomyocytes and primary neonatal rat ventricular cardiomyocytes were developed, with these *in vitro* cell models characterised and optimised, to enhance detection of anthracycline-induced cardiotoxicity. The current clinical consensus is to consider adult and paediatric anthracycline-induced cardiotoxicity within the same pathological process. Recognition of the developmental physiology of cardiomyocytes from birth to adulthood, and analysis of the key histological differences between paediatric and adult patients diagnosed with anthracycline-induced cardiotoxicity, indicate that they should be examined as separate entities. Furthermore, comparing the trends of late-onset anthracycline-induced cardiotoxicity between children and adults, the variation in cardiac biomarkers following anthracycline chemotherapy (higher sensitivity of cardiac troponins in adults, whilst natriuretic peptides have a higher sensitivity in paediatric patients), and differences in their respective responses to RAAS inhibitors, all strengthen support for this position (Lipshultz et al., 2002, Cardinale et al., 2006, Michel et al., 2020a, Michel et al., 2020b). The protocols developed and experimental designs provide an excellent platform for future studies, as these *in vitro* clinical models were optimised to replicate the physiological differences between adult and paediatric cardiotoxicities, whilst doxorubicin dosing schedules were modified to reflect clinically relevant drug concentrations, therefore enhancing clinical relevance.

The existing scientific literature demonstrates that RAAS is modulated by anthracycline chemotherapy and is implicated in the pathophysiology of cardiotoxicity. Histopathology studies of *in vitro* and *in vivo* cardiomyocytes exposed to doxorubicin have confirmed the presence of cellular hypertrophy and fibrosis in cardiac tissue (Olivetti et al., 1991, Bernaba et al., 2010). Identical myocardial changes have been detected in cardiac tissue exposed to angiotensin II, indicating that activation of the ACE/angiotensin II/AT1R axis may be responsible for anthracycline-induced cardiotoxicity (Hunter and Chien, 1999). Within this thesis, evaluation of angiotensin signalling in response to doxorubicin exposure demonstrated dose and time-dependent increases in AT1R gene expression in adult ventricular cardiomyocytes. These results therefore support the mechanistic hypothesis that a

relationship exists between anthracycline-induced cardiotoxicity and modulation of the angiotensin signalling pathway in isolated cardiomyocytes. Upregulation of the AT1R gene within these *in vitro* studies crucially demonstrates that doxorubicin has a direct effect upon the cardiomyocytes, rather than uniquely through systemic RAAS activation and regulation of cardiovascular haemodynamics. In addition, these findings have important implications for the treatment and prevention of anthracycline-induced cardiotoxicity, as therapies need to target the ACE/angiotensin II/AT1R axis, specifically interacting with the angiotensin II receptor.

To establish the aetiology and clinical implications of AT1R upregulation, the relationship between plasma angiotensin II concentrations, ACE genotyping, and anthracycline-induced cardiotoxicity were investigated. These analyses demonstrated no correlation between pre-chemotherapy systemic angiotensin II concentrations, the ACE genotype, and development of the cardiotoxicity phenotype in patients treated with anthracycline chemotherapy. These results appear to contravene the hypothesis that pre-existing elevations in circulating angiotensin II are attributed to inducing anthracycline cardiotoxicity, instead signifying that either the angiotensin II is produced locally within the myocardium to interact with AT1R, or that the presence of anthracyclines enhances the sensitivity AT1R to angiotensin II, as indicated by the increases in AT1R expression. Therefore, at present, these findings neither support the role for baseline plasma angiotensin II concentrations as a cause for anthracycline-induced cardiotoxicity, or as an isolated predictive factor for development of this phenotype in patients. Interestingly, angiotensin II concentrations were detected within the media of AC10 cardiomyocytes treated with doxorubicin, indicating the potential stimulation of local angiotensin II and intracardiac RAAS activation in response to anthracycline chemotherapy exposure (Baker et al., 1992). The next stage would be to complete prospective studies evaluating plasma angiotensin II concentrations and related biomarkers, such as serum angiotensin converting enzyme and angiotensin I, both during and after anthracycline chemotherapy. This would not only strengthen understanding of the angiotensin signalling hypothesis within cardiotoxicity, but also help to discern a direct relationship between cardiac anthracycline exposure and sequential RAAS activation.

Moving further upstream within the RAAS pathway, comparison of ACE genotyping from patients identified with and without the cardiotoxicity phenotype was completed. This series

of experiments were designed to investigate whether the ACE/angiotensin II/AT1R axis is modulated by ACE gene expression in the context of anthracycline-induced cardiotoxicity. Previous studies have shown that the ACE genotype is associated with the development of hypertension, ischaemic heart disease and heart failure (Cambien et al., 1992, Schunkert et al., 1994, Liu et al., 2021). This represents the first study evaluating the relationship between the ACE genotype and anthracycline-induced cardiotoxicity in patients. Comparison between patients diagnosed with anthracycline-induced cardiotoxicity and those without the cardiotoxicity phenotype, revealed that patients with cardiotoxicity were more likely to have the Deletion genotype. These differences, observed within a small sample size, were however not found to be statistically significant. In addition to complete larger scale studies to evaluate the ACE genotype in patients with anthracycline-induced cardiotoxicity, single nucleotide polymorphisms specific to anthracycline pharmacokinetics have been identified within the literature and necessitate further investigation, given that drug pharmacokinetics have significant effects on the cardiotoxic effects of anthracyclines (Findlay et al., 2019). Given the strength of these results, baseline ACE genotyping in cancer patients is currently not able to predict the development of late-onset cardiotoxicity, however, measurement of serum ACE levels before, during and after anthracycline chemotherapy may reveal positive trends in ACE production, indicating RAAS stimulation is caused by myocardial anthracycline exposure.

A continuous theme within this thesis was integrating the pre-clinical observations into the cardio-oncology clinics. Historically, cardio-oncology has been concerned with managing the consequences of 'off-target' chemotherapy effects, best demonstrated with the diagnosis and treatment of both subclinical and symptomatic anthracycline-induced cardiotoxicity. Following improvements to cardiotoxicity detection and surveillance, together with the emergence of new cardio-oncology guidelines, the speciality strongly advocates the clinical application of primary prevention strategies (Lyon et al., 2022). The PROACT clinical trial represents an excellent example, taking the strong evidence base supporting the use of ACE inhibitors within cardiovascular disease, and combining this with preclinical data demonstrating reductions in reactive oxygen species and oxidative stress responses observed *in vivo* with angiotensin II inhibition (Münzel and Keaney, 2001, Carrasco et al., 2021). Despite prospective clinical trial evidence advocating the rationale for ACE inhibitors as secondary prevention for anthracycline-induced cardiotoxicity (Cardinale et al., 2015), evidence for their

use as a primary preventative strategy remains limited and currently favours a biomarker-led treatment strategy (Kalam and Marwick, 2013, Cardinale et al., 2015, Janbabai et al., 2017).

6.2 Mitigation of anthracycline cardiotoxicity

A further example of translating research into the clinic was demonstrated with the analysis of blood pressure measurements in patients receiving anthracycline chemotherapy for breast cancer. This study was completed to evaluate the clinical implications of increased angiotensin signalling observed in the pre-clinical studies, and the consequences of raised circulating angiotensin II levels as a mediator for anthracycline-induced cardiotoxicity. The hypothesis was that RAAS activation, specifically increased angiotensin II synthesis, could directly modulate blood pressure and provoke hypertension in patients receiving anthracycline chemotherapy. If such a relationship was shown, these elevations in pre-chemotherapy blood pressure would further support ACEi prescribing to mitigate the effects of anthracycline-induced cardiotoxicity. Aware of the study limitations, including small patient numbers and confounding factors affecting clinical blood pressure recordings, this retrospective cohort found elevations in baseline diastolic blood pressure were significantly associated with developing anthracycline-induced cardiotoxicity (Philip et al., 2022). Although these measurements remained below the threshold consistent with a diagnosis of diastolic hypertension, they were found to be within the range considered as 'pre-hypertension'. These findings, supported by increases in angiotensin signalling expression, indicate the scope for sub-analyses within prospective clinic trials, where changes in blood pressure are measured throughout anthracycline chemotherapy and into the post-chemotherapy follow-up period. Overall, strengthening understanding of the underlying anthracycline-induced cardiotoxicity mechanisms can enhance primary prevention strategies, enabling a personalised approach to cardio-oncology care, with patient risk stratification and targeted prevention strategies aimed those consider 'high risk' of developing cardiotoxicity.

6.3 Cardiovascular risk assessment in patients with cancer

Cardiovascular risk assessment in patients with cancer aims to identify those at increased risk of cardiovascular complications relating to these cancer therapies, to then support these treatment and prevention strategies in promoting long-term cancer survival. The evaluation of cardiovascular risk profiles within three cancer groups (breast, prostate and lung),

reinforces the high cardiovascular risk factor burden within the oncology clinics. With quick application of the QRISK3 risk calculator and integration with the latest NICE guidelines (Duerden et al., 2015), this study demonstrated that over two-thirds of patients (72%) were considered at an increased cardiovascular risk. Due to the simplicity of this approach, cardiovascular risk can be quantified by the oncology team, to inform key decisions around oncology treatments and enable personalised surveillance strategies, whilst also supporting patient education regarding their cardiovascular risk (Curigliano et al., 2020, Lyon et al., 2022). Evidence taken from a UK cardio-oncology centre has proven that providing an individualised and targeted approach for patients, not only enhances rates of chemotherapy completion, but also reduces cardiovascular toxicities, as demonstrated by improvements in cardiac function and cardiovascular symptoms (Pareek et al., 2018). These observations are now supported within the new ESC cardio-oncology guidelines, which strongly emphasise this need for cardiovascular screening and risk prevention within high-risk patient populations (Lyon et al., 2022). A simple and effective approach identified within this thesis to immediately integrate these guidelines within clinical practice, is targeting improvements in primary prevention therapy prescribing and uptake for patients. In accordance with national clinical guidelines, patients identified with an elevated cardiovascular risk are recommended to receive primary prevention lipid-lowering therapy (Duerden et al., 2015). Unpublished regional primary care data has demonstrated that of 3152 patients with cancer (not including palliative care), only 45% of eligible patients were receiving lipid lowering therapy. This analysis within oncology clinics in secondary care revealed similar findings, with only 51% of eligible patients prescribed statin therapy. Beyond proven benefits for reducing ischaemic heart disease, pre-clinical studies have shown that statins possess additional anti-inflammatory and anti-oxidative properties, indicating their potential for prevention of anthracycline-induced cardiotoxicity (Henninger and Fritz, 2017). This hypothesis is supported with recent meta-analysis data demonstrating a strong association between statin therapy and the mitigation of anthracycline-induced cardiotoxicity. Within this analysis, rates of cardiotoxicity were found to be 50% lower amongst patients administered statins compared to those without (Obasi et al., 2021). By preserving LV ejection fraction during anthracycline chemotherapy, statins may provide clinicians with further therapeutic options for anthracycline-induced cardiotoxicity prevention. The results of several ongoing randomised

clinical trials are enthusiastically awaited to confirm their efficacy within this specific setting and provide new opportunities for pre-clinical studies (Omland et al., 2022).

Throughout this thesis, strong emphasis was placed upon understanding the mechanisms causing chronic anthracycline-induced cardiotoxicity. Chronic anthracycline-induced cardiotoxicity poses significant clinical challenges, especially identifying which patient groups are at risk, as even patients who received low cumulative anthracycline doses (<100mg/m^2) in the retrospective analyses were shown to develop the cardiotoxicity phenotype. Long-term cardiotoxicity surveillance is an area of advancing research, with the appropriate duration of long-term follow-up judged upon risk factors. These risk factors include cumulative anthracycline dose, mediastinal radiotherapy, previous cardiovascular co-morbidities, and cardiac impairment as determined by cardiac imaging, with the current consensus for long-term follow-up being between 1 and 5 years post-anthracycline chemotherapy (Armenian et al., 2016a, Curigliano et al., 2020, Lyon et al., 2022). A strong argument for implementing separate cardio-oncology surveillance pathways for paediatric and adult cancer survivors can be made, as demonstrated by comparing the paediatric and adult survivorship data. Cardinale et al. (2015) observed that 98% of cardiotoxicity cases in adults receiving anthracycline chemotherapy will have manifested before 12 months, with cardiac function remaining stable after 3 years (Cardinale et al., 2015, Meessen et al., 2020). By contrast, the paediatric patient study within this thesis demonstrated 66% of cardiotoxicity cases were identified after the first 12 months, with cardiotoxicity cases detected up to 19 years post-anthracycline chemotherapy completed. Findings from the histological analyses of explanted hearts from patients diagnosed with anthracycline-induced cardiotoxicity provide a plausible explanation for these differences. Evidence from the histology of paediatric patients with anthracycline-induced cardiotoxicity supports the 'Grinch syndrome' hypothesis, where reduced cardiac mass and cavity size are the result of significant cardiomyocyte losses incurred during anthracycline chemotherapy (Lipshultz et al., 2014). Therefore, as paediatric patients continue their cancer survivorship beyond adolescence, ongoing myocyte losses incurred through ageing means the threshold for diagnosing cardiotoxicity on echo will eventually be reached in these patients, and sooner than in healthy controls. Furthermore, the histological analyses identified significant grades of both interstitial and replacement fibrosis, indicating adverse changes in structural integrity of the myocardial tissues, meaning the heart as a

reduced contractile efficiency and therefore limited reserve capacity. In addition to providing valuable insights into the pathophysiology of cardiotoxicity, these conclusions can potentially justify extending the duration of long-term surveillance beyond 5 years, with the frequency of cardiac imaging determined by baseline and post-chemotherapy cardiovascular risk evaluation.

Finally, whilst administration of RAAS inhibitors prior to anthracycline chemotherapy may mitigate this adverse myocardial remodelling, Lipshultz et al. has suggested that the efficacy of ACE inhibitor therapy for the treatment of anthracycline-induced cardiotoxicity may be transient, with insufficient dosing or aldosterone escape mechanisms potentially limiting ACE inhibitor efficacy (Lipshultz et al., 2002). There therefore remains a strong clinical need to establish how the renin-aldosterone-angiotensin axis interacts with primary prevention therapy, and further clinical trials are required to provide strong recommendations on the appropriate duration of ACE inhibitor therapy.

6.4 Limitations and future directions

The *in vitro* models described and applied in this thesis provide an excellent platform for future research studies evaluating the relevant perturbations of anthracycline-induced cardiotoxicity. The AC10 cardiomyocytes were the predominant preclinical model applied for the analysis of growth kinetics and cellular viability helped identify the optimal experimental conditions for detecting doxorubicin-induced cardiomyocyte responses, with validation studies previous published in this field (Rockley, 2018, De Santis, 2020). A significant advantage of this cellular model is their ability to form a stable monolayer in culture, as demonstrated by the plateau in xCELLigence impedance values. This important *in vitro* observation means that AC10 cardiomyocytes can be applied for both acute and chronic toxicity studies evaluating structural remodelling in response to anthracycline exposure. Whilst the AC10 cardiomyocytes are therefore highly relevant for detecting structural cardiotoxicities when cultured *in vitro*, their pre-contractile state provides a notable limitation for analysing functional cardiomyocyte changes. To enhance and reinforce the research conclusions within this thesis, the contractile properties of patient-specific hiPSC-derived cardiomyocytes (hiPSC-CMs) would display strength as a clinically relevant model for identifying acute functional cardiotoxicities, especially as anthracycline-induced cardiotoxicity is expressed in terms of functional parameters within the cardio-oncology clinics (Burridge et

al., 2016, Magdy et al., 2018). The comparatively short lifespan of hiPSC-CMs in culture renders them limited to identifying the early stages of cardiotoxicity, rather than chronic anthracycline-induced cardiotoxicity. Therefore, it is recommend integrating the contractile and phenotypic properties of hiPSC-CMs, to the structural sensitivity of AC10 cardiomyocytes, to ensure a comprehensive and clinically relevant evaluation of anthracycline-induced cardiotoxicity.

To appraise the angiotensin signalling mechanisms and structural manifestations specific to anthracycline-induced cardiotoxicity, increases in AT1R gene expression in response to doxorubicin exposure were successfully demonstrated. To determine the strength of the angiotensin-doxorubicin relationship, using short interfering RNA (siRNA) to silence AT1R expression in cardiomyocytes would confirm, by absence, whether the morphological changes observed were indeed linked to the angiotensin signalling mechanism. Furthermore, the direct application of an ARB, with AT1R-specific binding, within the culture medium combined with subsequent structural and viability analyses of the cardiomyocytes throughout both the acute and chronic phases of doxorubicin exposure would again reinforce our conclusions. Future experiments administering varying doses of ACEi/ARB to determine their cellular cardioprotective effects, whilst simultaneously evaluating RAAS receptor and biomarker expression, would be strongly recommended. Therefore, determining exactly how these treatments mitigate cardiotoxicity, informing their optimisation in clinical practice, and creating potential for new cardioprotective treatments.

From a clinical perspective, detailed investigation of RAAS biomarkers, including measuring the variability of angiotensin II plasma concentrations and blood pressures, prospectively in patients throughout their chemotherapy treatment may further establish the clinical influences of the anthracycline-induced cardiotoxicity-angiotensin signalling relationship. As outlined within the biomarkers chapter, optimal sampling, measurement and parallelism studies for each of the constituents of the RAAS pathway, may reveal how angiotensin signalling in influenced throughout the course of chemotherapy and especially in response to ACE inhibitor therapy for anthracycline-induced cardiotoxicity prevention.

Finally, given the differences in stages of cardiomyocyte development and myocardial growth established between paediatric and adult patients, this research chose to evaluate paediatric and adult cardiotoxicities separately. In addition to contrasting cardiomyocyte proliferation

rates, the myocardium constitutes of differing cellular proportions for example fibroblasts and cardiac progenitor cells, which possess different properties when exposed to anthracyclines. Future lab-based studies are proposed to determine the responses to doxorubicin in both paediatric and adult cardiomyocyte models, with relevant dosing schedules designed to recreate drug pharmacokinetics and responses to ACE inhibitors, establishing differences in both acute and chronic responses to anthracyclines.

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Chapter 7. Appendices

Appendix A: Cardiomyocyte Cell Culture Protocol

This protocol describes the methods of sample preparation for the culture and differentiation of neonatal rodent cardiomyocytes. Aiming to keep the cardiomyocytes in the logarithmic growth phase.

Solutions (all from Sigma, storage temperature in brackets)

1. Phosphate-Buffered Saline (PBS) (room temp).
2. Complete Dulbecco's Modified Eagle Medium (DMEM) (4°C): 500ml incomplete DMEM (4°C), 50ml FBS (-20°C), 5ml L-Glutamine (-20°C), 5ml Penicillin/Streptomycin (4°C).
3. 0.25% Trypsin-EDTA solution (-20°C).

Preparation and Sterilisation

All apparatus and liquids that come into contact with cultures or other reagents must be sterile.

- Prior to starting, DMEM and Trypsin-EDTA should be incubated to 37°C
- Turn on incubator containing rocker to warm to 37 °C

Method

- Neonatal rodent heart extraction performed under sterile conditions
- Hearts transferred immediately into a petri-dish containing 5ml PBS and put onto ice
- Prepare three petri-dishes
- Add 5ml PBS to two of the petri-dishes, and to the third add 5ml Trypsin-EDTA solution
- Sterilise fine dissection scissors and non-toothed forceps
- Ensure quick working to minimise cellular death

1. Using forceps, initially rise neonatal heart in PBS to remove excess blood and attached cells
2. Again using forceps, transfer heart to second petri-dish containing PBS
3. Remove aorta, pulmonary vessels and atria using fine dissection scissors

4. Rise from excess blood and cells in PBS solution
5. Transfer remaining heart containing ventricular cells to petri-dish containing Trypsin-EDTA solution
6. Using fine dissection scissors, dissect ventricular tissue transversely, aiming for fine slices of tissues
7. Return cells to Trypsin-EDTA solution
8. Aim for minimum of three neonatal rodent hearts to the Trypsin-EDTA solution to ensure adequate cell numbers
9. Transfer Trypsin-EDTA solution containing neonatal ventricular cells to pre-warmed incubator containing rocker
10. Ensure petri-dish is secure (using autoclave tape) to the plate and turn on the rocker to 1000
11. Leave cells incubated for 30 minutes to allow adequate time for Trypsin-EDTA to take effect
12. After 30 minutes, remove cells from incubator and return to sterile hood
13. Pipette 5ml Trypsin-EDTA solution containing ventricular cardiomyocytes, sieving cells through a 70µm cell strainer into non-skirted universal tube
14. Rinse petri dish with 2ml DMEM to collect remaining cells and rinse through sieve to maximise collection of ventricular cells
15. Centrifuge for 5 minutes at 400 x g
16. Remove supernatant
17. Re-suspend in 4ml fresh DMEM, gently shaking and pipetting up & down to dislodge cells and break up aggregates
18. Count cells with a haemocytometer
19. Transfer ventricular cardiomyocytes suspended in DMEM into 6-well plate at the required cell density, adding further DMEM to a total of 2ml per well
20. Incubate for 30 minutes at 37°C
21. After 30 minutes, transfer contents of each well into adjacent well, this aims to remove the fibroblasts which adhere early to the base of the wells
22. Replace 6-well plate with ventricular cardiomyocytes into incubator at 37°C
23. After every 24 hours, change the media

24. Ventricular cardiomyocytes adhere to the base of the well and show signs of beating spontaneously after 36-48hrs
25. Beating cardiomyocytes can be maintained for between 3-4 weeks in DMEM

Appendix B: Candidate reference gene ranking

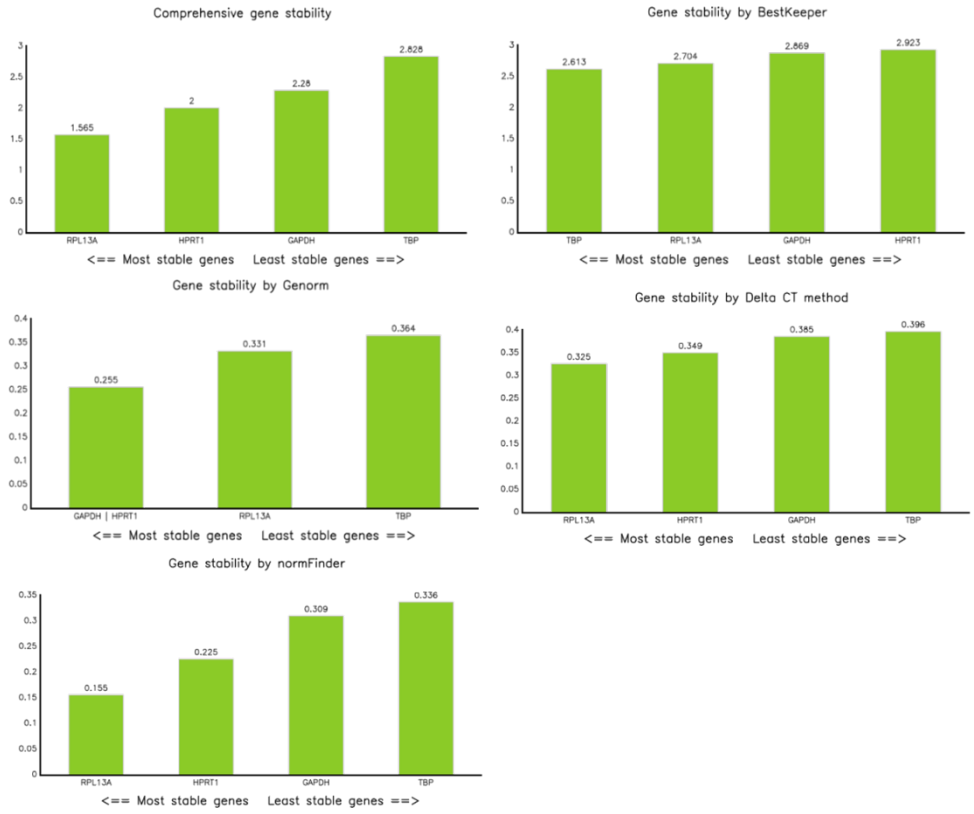

Figure 7.1 Ranking of the four candidate reference genes using different algorithms

Figure 7.2 Expression stability data of candidate reference genes by BEST KEEPER

	GAPDH	HPRT1	TBP	RPL13A
n	15	15	15	15
geo Mean [CP]	29.60	28.96	29.44	20.31
AR Mean [CP]	29.79	29.15	29.59	20.54
min [CP]	24.93	24.44	24.94	16.11
max [CP]	34.78	34.31	34.23	24.95
std dev [+/- CP]	2.87	2.92	2.61	2.70
CV [% CP]	9.63	10.03	8.83	13.16
min [x-fold]	-25.47	-22.90	-22.52	-18.29
max [x-fold]	36.20	40.72	27.82	24.98
std dev [+/- x-fold]	7.31	7.58	6.12	6.52

Appendix C: PROACT and PROACT PLUS: DNA Sampling Protocol

INSTRUCTIONS:
BLOOD SAMPLES:

- Mix samples thoroughly for 2 minutes to prevent clotting
- Sampling of 5mls venous blood in EDTA (purple) bottles
- Samples must be labelled with patient identifiers (surname, individual name, date of birth, NHS number)

DNA SWAB SAMPLES: Isohelix – DNA buccal swabs (SK-1S/MS-01)
Where patients have provided additional consent, a buccal swab should be collected at baseline.
Store at room temperature. Single Use. Only use if wrapper remains intact.
Ideally, the patient should not eat, drink, smoke, brush teeth or use mouthwash for 2 hours prior to the swab being taken.

1. Open the packet and remove swab, care being taken not to touch the white swab head to maintain aseptic technique
2. Patient to insert the swab into their mouth and rub firmly against the inside of the cheek or underneath lower and upper lip. For standard DNA collection rub for 1 minute.
   Important - Use reasonable, firm and solid pressure
3. Place the swab back into the tube. Do not touch the swab head with your fingers
4. Place your thumbnail in the small groove set in the handle, then snap the handle in two be bending to one side. Letting the swab head fall into the tube
5. Seal the tube securely with the cap provided
6. Label the sample with the patient's trial number and initials

PROACT STUDY ID: _ _ _ _ DOB: _ _/ _ _ _ _(mm/yyyy)
Date collected: _ _ / _ _/ _ _ (dd/mm/yy)
Time collected: _ _ : _ _ (hh:mm, 24 hr clock)
Visit: Baseline
Sample type: BUCCAL SWAB

7. Place swab directly into dry transport tube or collection envelope, again labelling
8. Complete the buccal swab sample request form and include within the swab.

Sample Handling:
Samples can be transported at room temperature. Samples may be stored at room temperature if taken on the day they are to be sent or refrigerated overnight. If prolonged storage is necessary at least minus $20^{O}C$.

Appendix D: Immunofluorescence demonstrating mean intensity distribution of AT1R expression in AC10 cardiomyocytes

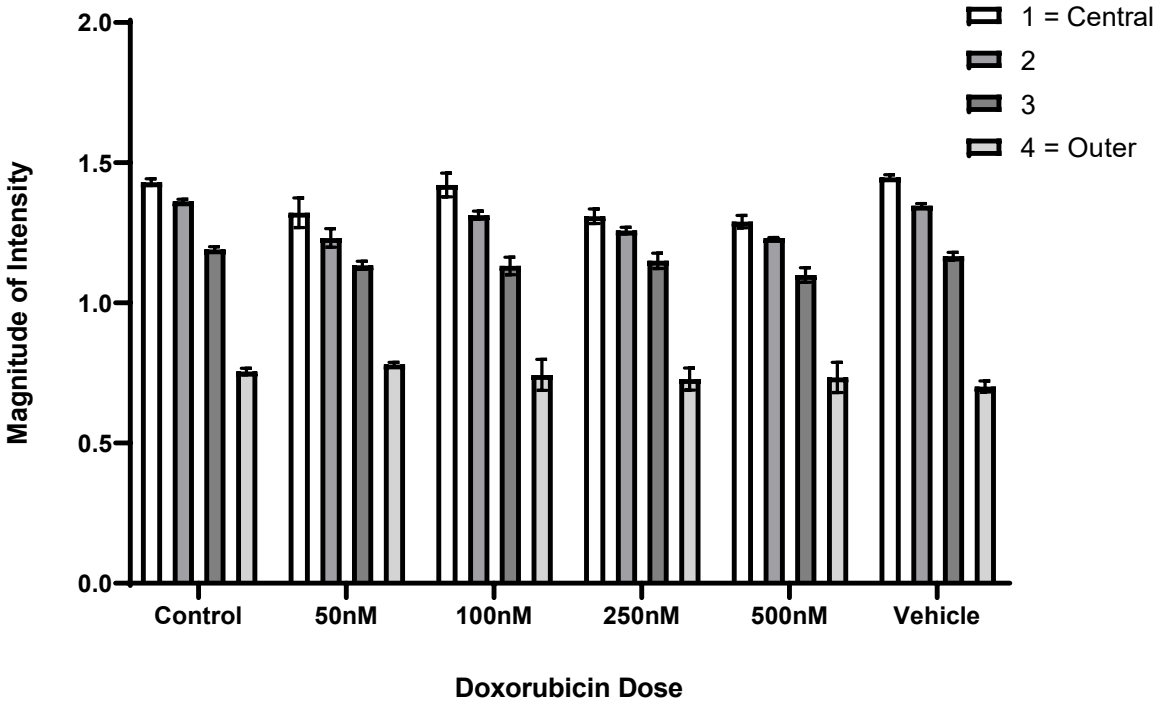

Figure 7.3 **Mean intensity distribution of AT1R expression in AC10 cardiomyocytes exposed to doxorubicin for 24 hours (± s.d., n=3)**

Appendix E: Publications and presented abstracts

- **Findlay, S.G**., Gill, JH., Plummer, R., De Santis, C., Plummer, C. (2019). Chronic cardiovascular toxicity in the older oncology patient population. *Journal of Geriatric Oncology*. 10:685-689
- De Santis C., **Findlay S.G.**, Gill JH., (2019). Poster Presentation and Abstract. Qualification of in-vitro Cardiac Cell Models for Preclinical Assessment of Oncology Drug-induced Cardiotoxicity. *European Cardio-Oncology Symposium*, Barcelona, Spain.
- **Findlay, S.G**., Plummer, R., Plummer, C. (2020). Cancer Immunotherapy and its potential cardiac complications. British Journal of Cardiology. 27:19-23
- S Findlay, J.H Gill, R Plummer, C.J Plummer. (2020). Published Abstract. Role of angiotensin-signalling in anthracycline-induced cardiotoxicity, ***European Heart Journal***, Volume 41, Issue Supplement_2, November 2020, ehaa946.0883
- **Findlay S.G.**, Gill JH., R Plummer, C.J Plummer. (2020). Poster Presentation. Role of angiotensin-signalling in anthracycline-induced cardiotoxicity. *European Society of Cardiology Conference -Oncology Symposium*, Digital Experience.
- **S Findlay**, C J Plummer, R Plummer, J H Gill. (2021). Published Abstract. The role of angiotensin signalling in anthracycline-induced cardiotoxicity, ***European Heart Journal***, Volume 42, Issue Supplement_1, October 2021, ehab724.2882,
- **Findlay S.G.**, Gill JH., R Plummer, C.J Plummer. (2021). Poster Presentation. The role of angiotensin signalling in anthracycline-induced cardiotoxicity. *European Society of Cardiology Conference -Oncology Symposium*.
- Philip, L.J., **Findlay, S.G**., Gill, JH. (2021). Baseline blood pressure and development of cardiotoxicity in patients treated with anthracyclines: A systematic review. *Int J Cardiol Cardiovasc Risk Prev.* 15:200153.

Review article published in the Journal of Geriatric Oncology, Sept 2019

Review > J Geriatr Oncol. 2019 Sep;10(5):685-689. doi: 10.1016/j.jgo.2019.01.018. Epub 2019 Feb 5.

Chronic cardiovascular toxicity in the older oncology patient population

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Affiliations + expand

PMID: 30733001 DOI: 10.1016/j.jgo.2019.01.018

Abstract

Survivorship statistics demonstrate that the incidence of cancer continues to rise worldwide, with a further 60% increase in diagnoses predicted by 2030 attributed to lifestyle risk factors, screening programmes resulting in earlier diagnosis but also the changing demographics of the population. More than a third of new cancer diagnoses and almost half of cancer survivors are now aged 70 years or older. Despite this increasing incidence, worldwide five-year cancer survival rates have improved significantly over the past two decades. After cancer, cardiovascular disease is the second most common cause of death in developed countries. With continued improvements in overall prognosis, patients with cancer have an increased exposure to cardiovascular risk factors resulting in higher cardiovascular morbidity and mortality, particularly in older patients. This relationship between cancer and cardiovascular disease is not surprising as they share the common risk factors of aging, smoking, obesity, and poor diet. In this review, we discuss the toxicity of cancer treatments on the cardiovascular system, particularly in older patients. We focus primarily on radiotherapy and anthracycline chemotherapy because of their chronic adverse effects and appraise approaches toward the detection and treatment of this toxicity to maximise survival and quality of life of older patients with cancer.

Abstract & Poster presented at the European Cardio-Oncology Symposium, Barcelona, 2019

Qualification of in vitro cardiac cell models for preclinical assessment of oncology drug-induced cardiotoxicity

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Cardiotoxicity is a major cause of drug attrition and life-threatening complication of many anticancer therapies. Current preclinical methodologies for identification of drug-induced structural and/or functional cardiotoxicity and their underlying mechanism are sub-optimal, involving either non cardiac cell lines expressing specific ion channels, or ex-vivo cardiac tissues with limited utility for longer-term analyses and clinical translation. The emergence of innovative technologies combined with the use of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM), with the ability to synchronously contract in-vitro, revolutionised identification of drug-induced cardiotoxicity. However, major limitations to this approach are their complexity, specialised culture conditions, high cost and subsequent limitations for assessment of drug-induced cardiotoxicity. One resolution is incorporation of cardiac cell lines into the initial screening paradigm. The study objective was to evaluate the responsiveness of current cardiac cell-lines to both structural and functional cardiotoxicants, specifically the human ventricular cardiomyocyte cell-line, AC10, and the murine atrial cell-line, HL-1, relative to both primary neonatal rodent cardiomyocytes and hiPSC-CM. Real-time assessment of pathophysiological changes were determined by impedance-based in vitro methodologies (xCELLigence RTCA), with known cardiac therapeutics and the histone deacetylase inhibitor (HDACi) class of drugs being evaluated in this context. The AC10 cell-line, although non-contractile, was able to detect drug-induced structural changes. The HL-1 cell-line was also able to detect structural cardiotoxicants, and exhibited a contractile phenotype in vitro, albeit non-uniformly and time-limited. This study indicates there is scope for inclusion of cardiac cell-lines alongside primary cells and hiPSC- CM in preclinical evaluation of drug-induced cardiotoxicity.

(Poster available at: https://eurocardio-onc.cme-cpd.org)

Qualification of *in-vitro* Cardiac Cell Models for Preclinical Assessment of Oncology Drug-induced Cardiotoxicity

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Northern Institute for Cancer Research, Newcastle University, UK

ecos European Cardio-Oncology Symposium

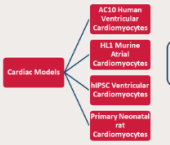
Newcastle University UK | Malaysia | Singapore

INTRODUCTION

Cardiotoxicity is a major complication of many anticancer therapies that impacts the quality of life and overall survival of patients. Preclinical models with improved ability to predict structural and functional cardiac liabilities are required to identify toxicological mechanisms, reduce clinical cardiotoxicity potential and identify therapeutic strategies to mitigate these life-threatening effects.

STUDY AIMS:

- Using impedance based real-time cell analysis (xCELLigence RTCA), evaluate the ability of different cardiac cell models to detect structural and functional drug-induced cardiotoxicity



Detection of cardiotoxicity in response to Histone deacetylase inhibitor class of drugs

- Several HDAC inhibitors have been introduced to the clinic or are currently in trial
- HDAC inhibitors demonstrate clinical cardiotoxicity
- **What is the involvement of HDAC sub-classes in HDACi-induced cardiotoxicity?**

METHODOLOGY

Fig 1. xCELLigence Real-Time Cell analysis and Contractility:

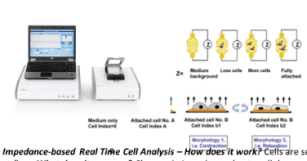


Fig.1 ***Impedance-based Real Time Cell Analysis – How does it work?*** Cells are seeded on an electrode coated plate. When a mild current is applied the attachment of the cells will impede the current flow. ***What does it measure?*** Changes in impedance due to cellular growth and morphology are recorded in real time. These can change in response to drugs or toxic insults.

Fig 2. Qualification of xCELLigence system to detect functional changes in cardiomyocyte contractility

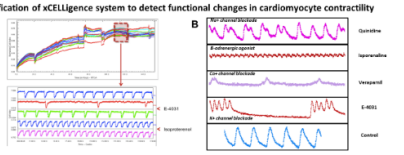


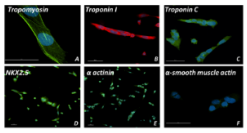
Fig 2. Changes in contractility in hiPSC-CM (A) and primary neonatal CMs (B) induced by cardioactive agents

RESULTS

HDAC inhibitor at clinically relevant concentrations induces structural changes in AC10 ventricular cardiomyocytes

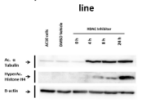
AC10 ventricular human cell line

Fig 3. Cardiac phenotype of AC10 cells



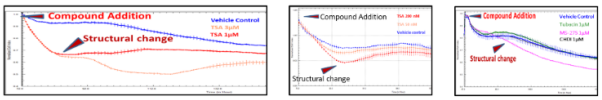
AC10 cells are positive for A. Tropomyosin, B. Troponin I, C. Troponin C, D. NKX2.5 and E. alpha-actin. Negative for F. alpha smooth muscle actin

Fig 4. TSA-mediated HDACi induces Time-dependent Histone and Tubulin acetylation in human AC10 cardiomyocyte cell line



AC10 cells at confluency were exposed to the pan-HDACi TSA (1 μM). Expression of acetylated histone H4 and acetylated α-tubulin protein was determined by western blot analysis

Fig 5. Inhibition of HDACs at sub-toxic concentrations induces structural change in AC10 cells



Sub-toxic concentration of TSA induced a decrease in cell index but not a decrease in cell number, suggesting cytotoxicity may depend on cell hypotrophy secondary to HDACi downstream effects

Selective inhibition of class I HDACs induces structural changes in AC10 cell line. Selective inhibition of class II HDACs does not induce significant cell index alterations.

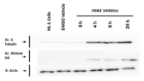
HDAC inhibitor at clinically relevant concentrations induces structural and partial functional changes in HL1 atrial cardiomyocytes

HL1 atrial murine cell line

Fig 6. Electrophysiology of HL1 cells

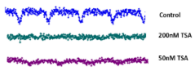
Compound	Mechanism	Detectable effect in HL-1 cell line using xCELLigence Cardio
Quinidine	Sodium channel blocker class Ia	✓
Disopyramide	Sodium channel blocker class Ia	X
Lidocaine	Sodium channel blocker class Ib	✓
Propaferone	Sodium channel blocker class Ic	X
Metoprolol	Selective B-adrenergic blocker (short acting)	X
Carvedilol	Non-selective α/B-adrenergic blocker (long acting)	X
Amiodarone	Potassium channel blocker	X
Verapamil	Calcium channel blocker	✓
E-4031	Experimental hERG blocker	✓
Isoprenaline	B-adrenoceptor agonist	✓

Fig 7. TSA-mediated HDACi induces Time-dependent Histone and Tubulin acetylation in HL-1 cell line



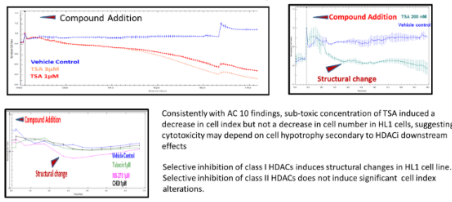
HL-1 cells at confluency were exposed to the pan-HDACi TSA (1 μM). Expression of acetylated histone H4 and acetylated α-tubulin protein was determined by western blot analysis

Fig 8. TSA-mediated HDACi induces functional changes in HL1 contractility



HL1 show functional changes in response to HDACi treatment. Loss of contractility is observed within 24h post drug exposure at 200nM.

Fig 9. Inhibition of HDACs at sub-toxic concentrations induces structural change in HL1 cells



Consistently with AC 10 findings, sub-toxic concentration of TSA induced a decrease in cell index but not a decrease in cell number in HL1 cells, suggesting cytotoxicity may depend on cell hypotrophy secondary to HDACi downstream effects

Selective inhibition of class I HDACs induces structural changes in HL1 cell line. Selective inhibition of class II HDACs does not induce significant cell index alterations.

hiPSC- CMs detect functional changes but not structural changes in response to HDAC inhibitor

Human iPS-derived cardiomyocytes

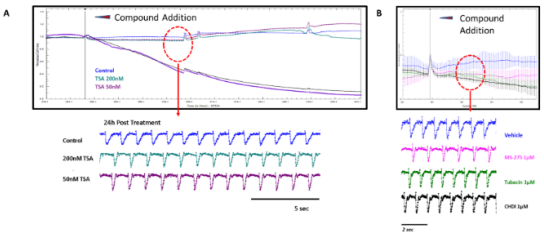


Fig 10. Trichostatin-A (TSA; pan-HDAC inhibitor) is associated with proarrhythmic and acute cytotoxic events detected using impedance assays in Cor.4U cells (A). Selective Class II HDACIs (Class IIa-Tubacin and Class IIb-CHDI) induce moderate decreases in cell index but no beating irregularities (A and B) ; changes in contractility frequency are observable upon treatment with class I HDACi (MS-275) (B).

HDAC inhibitor at clinically relevant concentrations induces functional and structural changes in primary rat cardiomyocytes

Primary rat-derived cardiomyocytes

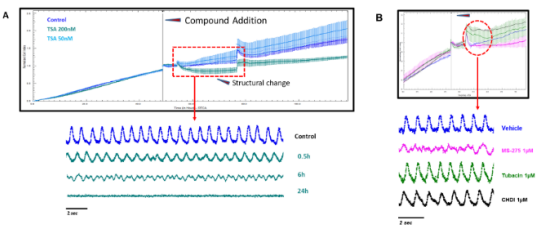


Fig 11. Trichostatin-A (TSA; pan-HDAC inhibitor) displayed a decrease in cell index at sub-therapeutic dose of 200nM in primary rat cells. Complete loss of contractility was achieved within 24hours from initial exposure (A). Selective Class II HDACIs (Class IIa-Tubacin and Class IIb-CHDI) did not induce changes in contractility, changes in contractility pattern are observable upon treatment with class I HDACi (MS-275) (B).

CONCLUSIONS

The combination of non-contractile primary cardiomyocytes and contractile cardiomyocytes offers a comprehensive model system for the detection of drug-induced structural and functional cardiotoxicity

Cardiac Model	Structural Screening	Functional screening
AC10 Human Ventricular Cell Line	YES	NO
HL-1 Murine Atrial Cell Line	YES	LIMITED
Human hiPS-CMs	LIMITED	YES
Primary Neonatal Rat CMs	YES	YES

The integration of different in-vitro models allowed to gain insights into HDACi-mediated cardiotoxicity

HDACi- induced Cardiotoxicity
HDAC inhibition causes both structural and functional aberrations to cardiac cells at sub-clinical drug concentrations
Class I HDACi induced detectable toxicity in the form of structural and functional perturbations
Class IIa and IIb HDACi did not cause detectable toxicity

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MRC Medical Research Council

itip

Case presentation and review article publication British Journal of Cardiology, 2020

Cancer immunotherapy and its potential cardiac complications

January 2020 Br J Cardiol 2020;27:19–23 doi:10.5837/bjc.2020.002 Leave a comment

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Authors: Simon G Findlay, Ruth Plummer, Chris Plummer Show details

First published online 29th January 2020

Recent advances in immune therapy for cancer have significantly improved the clinical outcomes of patients with advanced cancers, where prognosis has historically been very poor. With these new treatments have come new toxicities and, as the use of immunotherapy increases, we will see an increasing incidence of immune-related adverse events, with patients presenting as an emergency. It is important that all cardiologists, and other physicians who see these patients, are aware of life-threatening immune-related toxicities, in addition to their recommended investigation and treatment.

We describe a patient with acute cardiotoxicity secondary to immune therapy to illustrate the complexity of these adverse cardiovascular events, providing recommendations for screening, diagnosis and management.

Abstract Publication, European Heart Journal, 2020

Role of angiotensin-signalling in anthracycline-induced cardiotoxicity

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Anthracycline chemotherapy remains a key component of cancer treatment regimens in both paediatric and adult patients. A significant issue with their use is the development of anthracycline-induced cardiotoxicity (AIC), with subclinical AIC and clinical heart failure observed in 13.8% and 3.1% of patients, respectively. The major clinical complication of AIC is the development of late-onset cardiotoxicity, occurring several years after drug administration, presenting as life-threatening heart failure (HF). Determining the relationship between subclinical AIC and late-onset HF, strategies for mitigation of AIC, and impacts upon the cancer survivor population remains a complex challenge.

Administration of drugs targeting the angiotensin system, specifically angiotensin converting enzyme inhibitors (ACEi), have been reported to reduce AIC in the clinic. Whilst the therapeutic effect of ACEi in management of left ventricular systolic dysfunction and consequent HF is principally through optimisation of cardiac haemodynamics, the mechanism involved with mitigation of late-onset AIC several years after anthracycline exposure are currently unknown.

Using a variety of human cardiomyocyte in vitro models we have previously demonstrated induction of cardiomyocyte hypertrophy by angiotensin II and anthracyclines. Importantly, selective blockade of the angiotensin II receptor 1 (ATR1) on cardiomyocytes mitigated the anthracycline-induced hypertrophic response, implicating synergism between AIC and angiotensin signalling in cardiomyocytes.

Adult human ventricular cardiac myocyte AC10 cell-line were treated in vitro with a range of clinically relevant doxorubicin doses for clinically appropriate durations, with AT1 receptor gene expression evaluated using semi-quantitative PCR.

Our results confirm a positive correlation between clinically-relevant concentration of doxorubicin and induction of gene expression of ATR1 in AC10 cells, with up to 200% increases in ATR1 expression observed. Maximal doxorubicin-induced gene expression being observed at 8 and 24-hours, respectively. These preliminary results agreeing with clinical exposure parameters for this drug with protein expression studies being optimised to support these gene expression study results.

Our preliminary studies also imply patients developing AIC carry a deleted polymorphism within intron 16 of the ACE gene and increased systemic levels of the ACE product angiotensin II, both with a known association to hypertrophic cardiomyopathy.

Taken together, these data support our mechanistic hypothesis that a relationship exists between AIC and modulation of the angiotensin signalling pathway in cardiomyocytes, involving structural cellular changes and asymptomatic cardiac hypertrophy. An elevation in angiotensin II levels, potentially through polymorphisms in ACE, could thereby exacerbate anthracycline-induced hypertrophy and promote the development of late-onset anthracycline-induced HF.

Poster Presentation, European Society of Cardiology Conference, 2020

Role of angiotensin signalling in anthracycline-induced cardiotoxicity

Findlay SG; Plummer CJ; Plummer R; Gill JH.

Translational and Clinical Research Institute, Newcastle University, UK

INTRODUCTION

Anthracycline chemotherapy remains a key component of cancer treatment regimens in both paediatric and adult patients. A significant issue with their use is the development of anthracycline-induced cardiotoxicity (AIC), which can occur several years after anthracycline therapy, presenting as life-threatening heart failure. Angiotensin converting enzyme inhibitors (ACEi) reduce development of AIC.[1] Genetic polymorphisms within the ACE gene are associated with cardiomyopathy and left ventricular hypertrophy, with elevated ACE activity associated with a deletion polymorphism. However, the mechanism responsible for ACEi-mediated mitigation of late-onset AIC and genetic predisposing factors are currently unknown.

STUDY AIMS: The overall aim of this study was to investigate the involvement of Angiotensin II signalling in anthracycline-induced cardiotoxicity.

- Evaluating the effect of anthracycline exposure upon expression of Angiotensin Receptor 1 (ATR1) in a preclinical human ventricular cardiomyocyte cell line model
- Assessing whether the insertion/deletion (I/D) polymorphism (287 bp) in intron 16 of the ACE gene is a genetic risk factor for AIC, promoting anthracycline-induced cardiac hypertrophy

METHODOLOGY & RESULTS

Figure 1: AC10 human ventricular cardiomyocyte cell line expresses a cardiac phenotype. AC10 cells are positive for A) Tropomyosin, B) Troponin I, C) Troponin C, D) NKX2.5 and E) α-actin. Negative for F) α-smooth-muscle actin

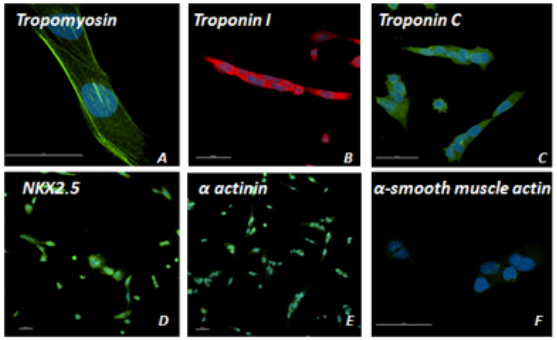

Figure 2: Effect of doxorubicin exposure upon expression of AT1R in AC10 cells *in vitro*. Exposure to clinically-relevant doxorubicin concentrations (< Cmax) induces time and concentration dependent increases in ATR1 gene expressions, determined by RT-PCR. Maximal expression being observed following 24hr drug exposure.

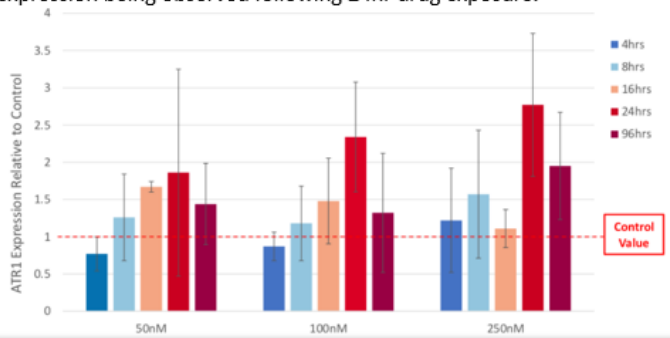

Figure 3: Presence of insertion/deletion polymorphism of ACE gene in patients treated with anthracyclines. DNA extracted from buccal swabs of cancer patients treated with anthracyclines analysed for 287bp insertion/deletion in intron-16 of ACE gene, relative to prevalence in general population.

ACE Gene Polymorphism	Cardiotoxicity Clinic (n=6)	Prevalence General Population[2]
D/D	17%	30.5%
I/D	83%	47%
I/I	0%	22.5%

CONCLUSIONS

- Exposure to doxorubicin induces angiotensin receptor 1 (ATR1) expression in the adult human ventricular cardiomyocyte AC10 cell line
- Doxorubicin-induced expression of ATR1 in conjunction with the presence of a genetic deletion polymorphism within intron 16 of ACE and associated elevated systemic angiotensin II levels may facilitate development of anthracycline-induced hypertrophy and heart failure
- Treatment with ACE inhibitors in this context may reduce or mitigate anthracycline-induced cardiotoxicity

References
1. Cardinale (2006)
2. Staessen (1997)

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Abstract Publication, European Heart Journal, 2021

The role of angiotensin-signalling in anthracycline-induced cardiotoxicity

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[3] Northern Centre for Cancer Care, Freeman Hospital, Newcastle upon Tyne Hospitals NHS Foundation Trust
[4] Department of Cardiology, Freeman Hospital, Newcastle upon Tyne Hospitals NHS Foundation Trust

Anthracyclines (e.g. epirubicin, doxorubicin, daunorubicin) are widely used for the treatment of adult and paediatric cancers. Despite their therapeutic efficacy, anthracyclines are associated with both acute and late-onset cardiac toxicities. Meta-analyses report an overt cardiotoxicity incidence of 6.3%, whilst sub-clinical cardiotoxicity incidence is 17.9% (1). Angiotensin converting enzyme (ACE) inhibitors are used to treat anthracycline-induced cardiotoxicity (AIC) (2) and despite their efficacy being well studied for the treatment of heart failure, hypertension and post-acute coronary syndromes, their mechanism(s) for treating and preventing AIC remain unknown.

Using in vitro cardiomyocytes, we evaluated the angiotensin signalling mechanisms stimulated by doxorubicin chemotherapy, applying quantitative PCR, immunofluorescence and real-time cell analysis technologies.

In vitro adult human ventricular cardiomyocytes (AC10 cell line) treated with clinically relevant sub-toxic concentrations of doxorubicin, demonstrate a dose and time-dependent increase in angiotensin II type-1 receptor (AT1R) gene expression. Maximal AT1R expression was observed after 24 hours' exposure at 250 nanomolar (nM), with qPCR recording up to 13-fold increases in expression relative to control (figure 1). Consistent with gene expression studies, doxorubicin also induced expression of AT1R at the protein level, with immunofluorescence imaging displaying upregulation of AT1R in association with doxorubicin concentrations up to 500nM (figure 2). Western blot results also support the induction of AT1R, however no relationship was observed between either doxorubicin concentration or drug exposure time.

Cellular growth and morphological changes of cardiomyocytes in response to clinically relevant doses of doxorubicin treatment were evaluated with real-time cell analysis using

impedance-based xCELLigence technology. During the early phases of doxorubicin exposure, an increase in cell size was observed, whilst experiments modelling the pharmacokinetics and serial half-lives of doxorubicin demonstrated reversibility of doxorubicin-induced cardiomyocyte injury following drug elimination.

These data support the mechanistic hypothesis that a relationship exists between AIC and modulation of the angiotensin signalling pathway in cardiomyocytes. We demonstrate that cardiomyocyte exposure to doxorubicin induces AT1R gene and protein expression, whilst doxorubicin-induced cardiomyocyte injury displays reversibility following drug elimination. Genetic polymorphisms within the ACE gene have been associated with cardiomyopathy and left ventricular hypertrophy. Our research now provides the platform to ascertain whether the ACE genotype contributes to heart failure from AIC, and whether an elevation in pro-hypertrophic angiotensin II levels could exacerbate anthracycline-induced hypertrophy and promote the development of late-onset anthracycline-induced heart failure.

Poster Presentation, European Society of Cardiology Conference, 2021

The role of angiotensin signallingin anthracycline-induced cardiotoxicity

Findlay SG; Plummer CJ; Plummer R; Gill JH. ***Contact Details: Dr Simon Findlay*** *simon.findlay@ncl.ac.uk* Translational and Clinical Research Institute, Newcastle University, UK

BACKGROUND

Anthracyclines are widely used for the treatment of adult and paediatric cancers. Despite their therapeutic efficacy, anthracyclines are associated with both acute and late-onset cardiac toxicities. Meta-analyses report an overt cardiotoxicity incidence of 6.3%, whilst sub-clinical cardiotoxicity incidence is 17.9%.

The mechanism(s) of angiotensin converting enzyme (ACE) inhibitors, for the treatment and prevention of anthracycline-induced cardiotoxicity remain unknown.

OBJECTIVES

- **Establish ACE inhibitor mechanisms for treating and preventing anthracycline-induced cardiotoxicity (AIC)**
- **Evaluate dose and time-dependent angiotensin II receptor type-1 (AT1R) gene and protein expression**
- **Examine cellular growth and morphological changes of cardiomyocytes in response to doxorubicin**

METHODS

- *In vitro* adult ventricular cardiomyocytes (AC10 cell line) were treated with clinically relevant doxorubicin concentrations
- Angiotensin signalling mechanisms were evaluated using RT-qPCR, immunofluorescence and real-time xCELLigence cell analysis technologies

RESULTS

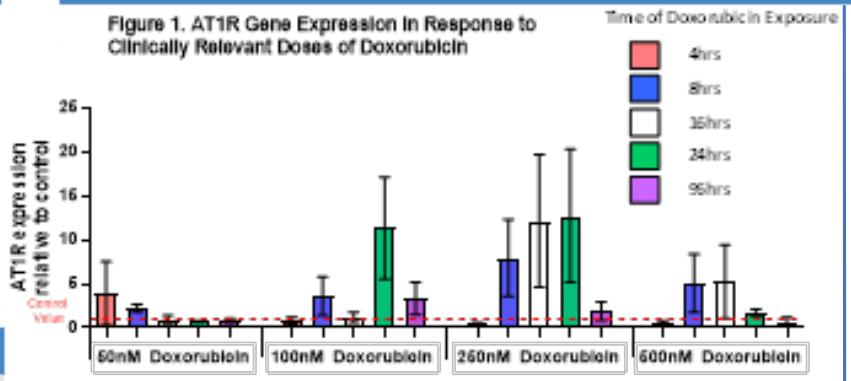


Fig 2. Immunofluorescence imaging AT1R expression in AC10 cardiomyocytes after doxorubicin exposure

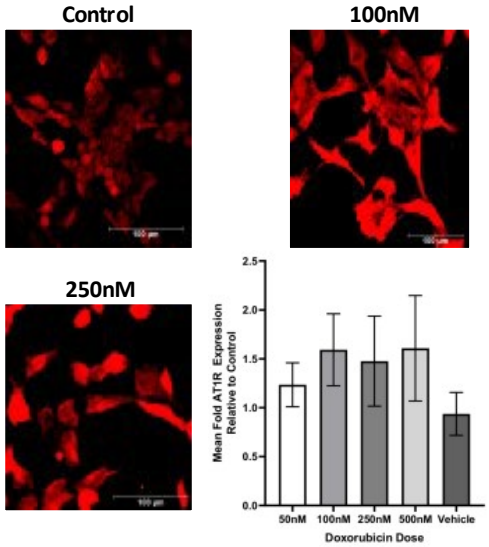
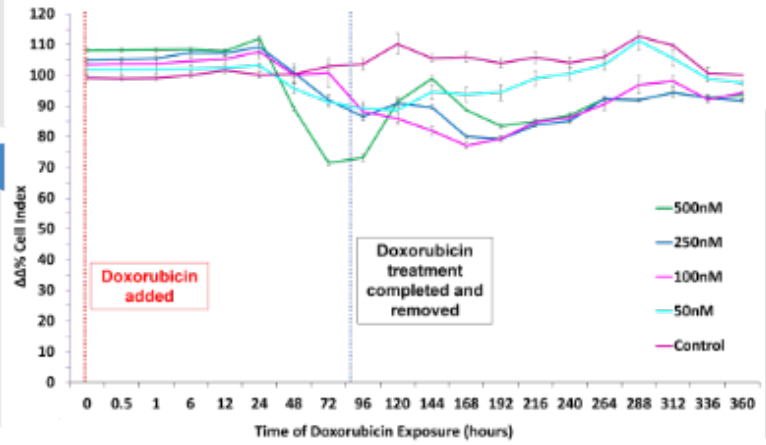


Fig 3. Impedance-based real-time cell analysis demonstrating reversibility of doxorubicin-induced cardiomyocyte injury following drug elimination



CONCLUSIONS

- **Doxorubicin induces AT1R gene and protein expression in cardiomyocytes**
- **Doxorubicin-induced cardiomyocyte injury displays reversibility following drug elimination**
- **These data support the mechanistic hypothesis that a relationship exists between AIC and modulation of the angiotensin signalling pathway in cardiomyocytes**

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