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Evaluation of novel biomarkers in the detection of chemotherapy-mediated cardiac and kidney injury

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A thesis submitted to

University of Dublin, Trinity College

for the degree of

Doctor of Philosophy

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I. Declaration

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II. Abstract

Chemotherapeutic agents are associated with a risk for potential cardiac and kidney injury, however diagnostic techniques currently used to assess cardiac function lack sensitivity, whilst those used to evaluate kidney function display significant variability. Recent studies have suggested that chemotherapy can cause cardiac myocyte injury, myocardial muscle stretch and cardiac remodelling in addition to kidney injury which can increase renal biomarker levels and decrease estimates of glomerular filtration rate (eGFR) and creatinine clearance (CrCl). The purpose of this study was to evaluate the cardiac biomarkers high sensitive cardiac troponin-I (hs-cTnI), amino-terminal pro-B-type natriuretic peptide (NT-proBNP), galectin-3 and to develop an assay for aldosterone using mass spectrometry. This study also aimed to evaluate the renal biomarkers creatinine, cystatin C and neutrophil gelatinase-associated lipocalin (NGAL) and their diagnostic utility in Cockcroft-Gault (CG), Modified Diet in Renal Disease (MDRD) and Chronic Kidney Disease (CKD-EPI) eGFR formulae in the assessment of kidney function, including in the application of chemotherapeutic drug dosing.

Patients treated with anthracycline agents demonstrated significant increases in hs-cTnI during the course of chemotherapy, which contrasted with comparable measures in patients receiving other chemotherapeutic regimens. This result suggests that anthracycline treatment induces cardiac myocyte damage. The relatively comparable measures of NT-proBNP, galectin-3 and aldosterone observed in patients receiving anthracycline agents suggests that myocardial remodelling was not evident during chemotherapy. The development of a novel liquid-chromatography tandem mass spectrometry (LC-MS/MS) assay facilitated the quantification of aldosterone in patient samples with lower concentrations than detectable using current methods.

Creatinine levels were stable in the oncology study cohort indicative of stable kidney function during chemotherapy. In contrast, elevated measures of cystatin C were identified when compared with a normal population both prior to and during treatment, suggestive of a malignancy-mediated influence which could diminish the clinical utility of cystatin C in assessing kidney function in this oncology population. Estimations of kidney function in the study cohort using CG as per pharmacy drug-dosing protocols, MDRD as reported by most diagnostic laboratories, and the newly advocated CKD-EPI formulae demonstrated significant differences when compared. This discrepancy leads to clinically relevant discordance in the interpretation of kidney function. CG and CKD-EPI demonstrated considerable differences in the doses of carboplatin calculated. As research has alluded to a closer association between CKD-EPI and the gold-standard method of measured GFR, the findings in this study highlight inaccuracy in current dosing protocols.

This study demonstrates a means of identifying cardiac and renal injury using biomarkers which can be assessed for the timely detection of organ damage, which has beneficial implications for future clinical trials aimed at minimising the adverse effects of chemotherapy. Furthermore, the discrepancy highlighted between renal assessment formulae in this study promotes the adoption of CKD-EPI SCr for the assessment of renal function and dosing of chemotherapeutic agents.

III. Acknowledgements

Firstly, I would like to sincerely thank my supervisor Dr Maria Fitzgibbon for her dedication, advice and guidance without whom this thesis would not have been possible. Your support was very kindly appreciated.

I would like to thank Abbott Diagnostics for funding support.

I would like to thank all of my colleagues in the Department of Clinical Chemistry and Diagnostic Endocrinology. I must especially thank Marguerite, Keith, Graham, Peadar and Rachel for their advice and guidance. To Steph, thank you so much for your help throughout the course of this project. Your advice on all things PhD-related has been invaluable.

To all the staff in specimen reception, thank you for your help during my project. To the nurses and staff of the Oncology/Haematology wards, thank you for your support during the study.

I must sincerely thank each patient who took part in this study, their dedication and willingness to take part in this clinical research was admirable and very kindly appreciated.

To Ray, Darina and Alan, thank you for all of your help and support, it was very kindly appreciated.

To Sean, I cannot thank you enough for your endless support during this study, you brightened up each day.

I would like to thank my family who have provided endless support through the years. I have to thank my Mam RIP, who always supported me every step of way. I dedicate this thesis to my Mam. To my Dad, thank you for all of your guidance, support and advice which has truly served me well over the years. I owe you a lot. To my sisters, Louise and Orla, you have both given me great support and have helped me in many ways, so much so that I will always be very grateful.

I am eternally grateful and feel blessed by the support I have received from everyone. A sincere thank you to everyone for making this work possible.

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VII. Abbreviations

ACE	Angiotensin converting enzyme
ACS	Acute coronary syndromes
AFP	Alpha fetoprotein
AKD	Acute kidney disease
AKI	Acute kidney injury
ANOVA	Analysis of variance
β	Beta
BMI	Body mass index
BNP	B-type natriuretic peptide
BSA	Body surface area
CA 125	Cancer antigen 125
CA 15-3	Cancer antigen 15-3
CA 19-9	Cancer antigen 19-9
CEA	Carcinoembryonic antigen
CG	Cockcroft-Gault
CG-CrCl	Cockcroft-Gault based creatinine clearance
CHF	Congestive heart failure
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology collaboration
CLSI	Clinical laboratory and standards institute
CrCl	Creatinine clearance

CREC	Cardiac review and evaluation committee
⁵¹ Cr-EDTA	Chromium EDTA
CSH	Charged surface hybrid
cTnI	Cardiac troponin-I
CV	Coefficient of variation
CysC	Cystatin C
DCM	Dichloromethane
δ	Delta change
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
ECHO	Echocardiogram
EF	Ejection fraction
eGFR	Estimated glomerular filtration rate
EQA	External quality assurance
ERM	European reference material
ESI	Electrospray ionisation
FDA	Food and Drug Administration (US)
FM	Final Measurement
FWHM	Full width-half maximum
GFR	Glomerular filtration rate
hCG	Human chorionic gonadotrophin
HF	Heart failure
HM	High mass resolution

HPLC-MS/MS	High performance liquid chromatography tandem mass spectrometry
hs-cTnI	High sensitive cardiac troponin-I
hs-cTnT	High sensitive cardiac troponin-T
IDMS	Isotope dilution mass spectrometry
IQR	Interquartile range
KDIGO	Kidney disease improving global outcomes
KO	Knockout
LC	Liquid Chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LM	Low mass resolution
LOD	Limit of detection
LOQ	Limit of quantification
LV	Left ventricular
LVEF	Left ventricular ejection fraction
MAX	Mixed-mode anion exchange
MDRD	Modified Diet in Renal Disease
mGFR	Measured GFR
MI	Myocardial infarction
MRI	Magnetic resonance imaging
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid

MS	Mass spectrometer
MT	Mid-treatment
MTBE	Methyl tert-butyl ether
<i>m/z</i>	Mass to charge ratio
NACB	National Academy of Clinical Biochemistry
NEQAS	National External Quality Assessment Scheme
NGAL	Neutrophil gelatinase-associated lipocalin
NIST	National Institute of Standards and Technology
NT-proBNP	Amino-terminal pro-B-type natriuretic peptide
NTT	Naïve to treatment
P20	Proportion of values within 20% of the measured GFR
PCI	Percutaneous coronary intervention
PETIA	Particle-enhanced turbidimetric immunoassay
PFP	Pentafluorophenyl
PSA	Prostate specific antigen
QC	Quality control
RAAS	Renin-angiotensin-aldosterone system
RF	Renal failure
RIA	Radioimmunoassay
SCr	Serum creatinine
SLE	Supported liquid extraction
S/N	Signal to noise
SPE	Solid phase extraction

SRM	Standard reference material
TIC	Total ion count
TR	Tricuspid regurgitation
UMR	Unit mass resolution
UPLC	Ultra high performance liquid chromatography
URL	Upper reference limit
WT	Wildtype

VIII. Abbreviated drug nomenclature

ABVD	Doxorubicin, bleomycin, vinblastine, dacarbazine
ACT	Doxorubicin, cyclophosphamide, paclitaxel
ACTH	Doxorubicin, cyclophosphamide, paclitaxel, trastuzumab
CHEOP	Cyclophosphamide, doxorubicin, vincristine, etoposide, prednisolone, ranitidine
CMF	Cyclophosphamide, methotrexate, 5-fluorouracil
DA 3+10	Daunorubicin, cytarabine
Folfox	Oxaliplatin, calcium leucovorin, fluorouracil
5-FU	5-Fluorouracil
M-FLOX	Calcium leucovorin, oxaliplatin, 5-fluorouracil
R-CHOP	Rituximab, vincristine, doxorubicin, cyclophosphamide, prednisolone
TC	Docetaxel, cyclophosphamide
TCH	Trastuzumab, carboplatin, docetaxel
TEH	Docetaxel, cyclophosphamide, trastuzumab
T-FAC	Paclitaxel, fluorouracil, cyclophosphamide, doxorubicin
TH	Paclitaxel, trastuzumab
THP	Paclitaxel, trastuzumab, pertuzumab

Chapter 1

Introduction to biomarkers and their clinical utility in detecting chemotherapy-mediated organ injury

1.1 A prelude to the study

Chemotherapeutic agents and more recently developed targeted therapies provide efficacious therapeutic options for many malignancies. Notwithstanding their clinical utility, undesirable organ damage can arise following treatment which can significantly impact clinical outcomes and patient prognosis. However, the therapeutic efficacy, compounded by the need for using such agents in the appropriate clinical setting commonly outweighs the decision to avoid use. The symptoms which develop are often heterogenic in nature and challenging to predict and identify before significant, irreversible damage has developed. As such, early detection is critical to attenuating organ damage.

The field of oncology is constantly evolving and the development of novel diagnostic techniques accompanies this evolution. Novel biomarkers and novel applications may hold potential in the detection of chemotherapy-mediated organ damage and their evaluation may reveal benefit with considerable translational capacity into routine clinical practice. In cases where current diagnostics are deficient in their ability to identify subclinical damage and where gold-standard practices are unrealistic in routine assessment, such novel diagnostic approaches may facilitate earlier therapeutic intervention and organ protection, thereby promoting a more informed approach to the utilisation and monitoring of chemotherapy treatment.

1.2 The role of chemotherapy in cancer treatment

Cancer encompasses an abandonment of normal cellular function and an adoption of infinite, destructive capabilities, leading to tissue and organ damage of often irreparable measures (Hanahan & Weinberg, 2000, 2011). Often potentiated by a metastatic propensity, the considerable impact to tissues and organs warrants timely intervention to limit malignancy, which can have potentially fatal consequences if not detected or successfully treated.

Originating as a means of chemical warfare, chemotherapy has been demonstrated as an efficacious treatment modality for cancer (Chabner & Roberts, 2005) and is grouped into agent classes based chiefly on structure, cell cycle specificity and cytotoxic activity, which aim to kill cancer cells in a pattern abiding the fractional cell kill hypothesis. Chemotherapeutic agents often demonstrate cytotoxic efficacy for specific cell cycles. In an effort to target as many cancer cells as possible, strategies using multiple agents which interfere with cancerous cells of various cell cycle stages, termed combination chemotherapy are often favoured. Multi-modality treatment is also a frequently utilised strategy which further emphasises the potential options and therapeutic permutations available which can be selected to effectively treat each patient.

Improved molecular profiling and genome sequencing is leading to a new era of more personalised and targeted cancer treatments with improved efficacy in many cancers and reduced but not eliminated bystander organ toxicity (Jackson & Chester, 2015). Integrating these newer therapies into cancer treatments for the future is very costly and clinically challenging and traditional chemotherapeutic regimens remain the mainstay of most cancer treatment pathways.

1.3 The adverse effects of chemotherapeutic agents

While appreciated for their cytotoxic activity, an undesirable consequence of chemotherapy treatment which is increasing in awareness, incidence and prevalence is that of organ damage arising from the toxic effects of chemotherapeutic agents. By virtue of the toxic attributes of chemotherapeutics or

their metabolites, complexed with routes of administration which often leave neighbouring healthy cells susceptible to cytotoxic activity, chemotherapy-mediated organ injury can have undesirable consequences for organ function and overall prognosis. The prospect of toxicity is further heightened by combination strategies which may potentiate the toxic effects of the agents or modalities comprised therein, thereby restricting the therapeutic potential of such treatment approaches and influencing their applicability for use. Furthermore, there is a growing expectation that such toxic sequelae may increase in prevalence with time, owing to improved cancer survival rates and an aging of the population which will include that of cancer patients.

Chemotherapeutic treatment is frequently associated with common acute toxicities, which can have adverse implications for patients encompassing delays to future chemotherapeutic treatment and potentially impacting quality of life. Organs such as the heart and kidneys can manifest cellular damage which can progress from subtle changes in structure and function, often undetectable at subclinical stages using current detection techniques to more established, dysfunctional phenotypes which can severely impact organ function. As such, timely detection of organ injury is imperative for the safe provision of agents designed solely for their antineoplastic and cytotoxic activity.

1.4 Laboratory analysis of biomarkers of chemotherapy-mediated organ injury

Immunoassay and radioimmunoassay techniques are commonly utilised laboratory methods for the quantification of biomarker concentration. Immunoassay is based on the principle of antibody-antigen binding where one is labelled with a molecule capable of emitting light following a chemical reaction. The principle of radioimmunoassay (RIA) is based upon the radiolabelling of an antigen which is bound to a pre-determined amount of antibody. Upon addition of the patient sample, the antigen present in the sample competes with the radiolabelled antigen, prompting its displacement which can in turn be measured and used to calculate the concentration of the analyte in question. Both of these

method types have limitations; the use and disposal of hazardous radioactive materials in RIA (Ishikawa, 1987) and interferences using immunoassay (Stowasser & Gordon, 2006). Additionally, both techniques are recognised to have issues with assay specificity (Cartledge & Lawson, 2000; Keevil, 2013), with considerable discordance when both methods are directly compared (Pizzolo *et al.*, 2006; Schirpenbach *et al.*, 2006).

1.5 Biomarker analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The field of oncology has witnessed significant advances in recent decades, with the emergence of novel biomarker applications which could revolutionise the manner and timing in which organ injury is detected and monitored. Accompanying this development is the continual improvement of analytical technology to facilitate more rapid, sensitive and specific means of quantifying biomarkers, as demonstrated by the advent of liquid chromatography-tandem mass spectrometry (LC-MS/MS) which quantifies analytes based on their mass to charge ratio (m/z).

The quantification of analytes using LC-MS/MS is a multifactorial process. The first phase of analysis incorporates the preparation of samples, whereby the analyte of interest is separated from other nonessential sample constituents such as proteins. This is an effort to confer specificity for the analyte of interest whilst also minimising sample interference. Additional concentration of the samples can be performed where sensitivity is limited. Examples of sample preparation techniques include liquid-liquid extraction (LLE), supported liquid extraction (SLE) or solid-phase extraction (SPE). LLE involves the addition of an immiscible or partially soluble liquid phase to a second liquid phase to facilitate the extraction of the analyte of interest (Mazzola *et al.*, 2008). SLE utilises sample cartridges or 96-well plates with a diatomaceous earth layer to capture the analyte and allow its elution by the addition of a solvent such as dichloromethane (DCM) or methyl tert-butyl ether (MTBE) (Keevil, 2013). SPE captures the analyte on a

sorbent while interferences from sample matrices and other sample constituents are removed before the analyte is eluted (Keevil, 2013).

Liquid chromatography (LC), allows further separation of the analyte of interest from other sample constituents by passing the pre-prepared sample in combination with mobile phases through an LC column. The selection of LC column, in terms of column size, chemistry and particle dimensions are critical components to ensuring good assay performance. In the case of aldosterone analysis, such procedures provide the means to separate the analyte from isobaric compounds with the same m/z ratio (Keevil, 2013). The eluent containing the analyte of interest emerging from the LC system passes through a fine steel capillary in the ion source and is nebulized upon exit with either a positive or negative charge in a process known as electrospray ionisation (ESI).

The final component of the analytical process comprises the entry of the ions into the mass spectrometer where the ions from the analyte of interest are separated from other ions for quantification or qualification. The analytes proceed into the first quadrupole where a charge is applied to select the ion of interest whose m/z is known and where unwanted ions are removed. The precursor ion proceeds into the collision cell where the ions encounter the collision gas (argon) which is coupled with a kinetic energy to fragment the precursor ion. The fragmented ions enter the second quadrupole where a voltage is applied to select the predetermined quantifier and qualifier ions of known m/z ratio in multiple reaction monitoring (MRM) mode. A non-isotopically labelled internal standard is typically added to the patient sample at the outset and is utilised to verify the assay performance, accounting for differences during sample preparation. In summary, LC-MS/MS is a highly specific and sensitive analytical technique which is now considered the gold-standard methodology for steroid and other analysis.

1.6 The essential role of biomarkers in oncology

Cancer diagnosis, monitoring and treatment is becoming more personalised and targeted and overall, outcome is improving. Tumour diagnosis and staging is generally a histological diagnosis, with increasing focus on tumour biological markers and somatic mutations that may guide treatment strategies. Routine haematological and biochemical monitoring of patients undergoing chemotherapy for blood, liver, bone and kidney organ dysfunction is an essential part of treatment and management protocols. In addition tumour-specific biomarkers are increasingly playing a role in cancer detection and determining prognosis. Circulating tumour markers are considered as molecules which indicate the presence of a specific cancer and provide information as to tumour behaviour (Duffy, 2006, 2013). The commonly measured tumour markers, include prostate-specific antigen (PSA; prostate cancer), cancer antigen 125 (CA 125; ovarian cancer), cancer antigen 15-3 (CA 15-3; breast cancer), cancer antigen 19-9 (CA 19-9; pancreatic cancer), alpha-fetoprotein (AFP; hepatocellular, germ cell), human chorionic gonadotropin (hCG; germ cell, trophoblastic disease) and carcinoembryonic antigen (CEA; colorectal cancer). Serial tumour marker monitoring is used as an indicator of tumour responses to treatment, and may be used as an indirect measure of drug efficacy or toxicity.

1.7 Cardiac injury mediated by chemotherapeutic agents

The pivotal role of chemotherapy in oncology is challenged by the accompanying risk for cardiac injury, which is often difficult to detect and treat with significant implications for patient prognosis (Yeh & Bickford, 2009). A conclusive body of evidence has demonstrated that many chemotherapeutic agents are accompanied by the risk of cardiotoxicity, including anthracycline agents (doxorubicin), trastuzumab, cyclophosphamide and paclitaxel (Yeh & Bickford, 2009; Schlitt *et al.*, 2014). As the symptoms which can arise can lead to potentially significant levels of cardiac damage, patients receiving these potentially cardiotoxic chemotherapeutic agents are classified by the American College of Cardiology and the American Heart Association as a Stage A heart failure (HF) group

(Bonow, 2005). The advent of improving cancer survival rates has been met with suggestions of a potential increase in the number of cases of cardiac injury arising from chemotherapy treatment which warrant timely detection.

The continual improvement in the variety of chemotherapeutic options available have contributed to an increasingly diverse array of symptoms of cardiac injury (Schlitt *et al.*, 2014), which are made all the more complex by the disparate timing of presentation. Of the agents found to confer risk for cardiac injury however, anthracyclines feature predominantly in cardiotoxicity studies, as a result of their applicability in the treatment of many malignancies which include solid organ tumours (Group, 2005) and haematological malignancies (Azim *et al.*, 2010). Anthracycline-mediated cardiac injury is classified as Type I cardiotoxicity by virtue of the often irreversible nature of such complications (Raj *et al.*, 2014). Many studies have shown that cardiac injury can present acutely following agent administration, in an early-onset chronic-progressive fashion which develops within the first year after treatment (Cardinale *et al.*, 2015; Wang *et al.*, 2015) or as a late-onset chronic cardiotoxicity which presents over one year following treatment completion (Simbre *et al.*, 2001). Such injury can have a serious impact on the cardiovascular health of cancer patients and can encompass an extensive range of symptoms (Schlitt *et al.*, 2014), including decreased left ventricular ejection fraction (LVEF) (Cardinale *et al.*, 2015), cardiomyopathy (Jensen, 2002) and congestive heart failure (Von Hoff *et al.*, 1979; Pinder *et al.*, 2007). By virtue of the often irreversible nature of such effects, early detection is essential to allow for the prevention or attenuation of cardiac damage (Vejpongsa & Yeh, 2014).

Many studies have alluded to a dose-dependent nature behind the incidence of anthracycline-mediated cardiac injury (Minotti *et al.*, 2004; Bovelli *et al.*, 2010; Curigliano *et al.*, 2012), although evidence of cardiotoxicity using what would be considered safe doses of anthracycline agents have also been detected (Bristow *et al.*, 1978), which highlights the unpredictability of cardiac injury when using this agent. Prior anthracycline chemotherapy, patient age, other chemotherapeutic agents, radiation therapy and patient cardiovascular comorbidities may mediate the risk for cardiac injury using such cardiotoxic agents (Bovelli *et al.*, 2010).

Anthracyclines mediate their action through intercalation of DNA, free radical generation and inhibition of topoisomerase II α (Raj *et al.*, 2014). Anthracycline-mediated oxidative stress, free radical generation and individual genetic variants are factors considered causative of cardiotoxicity (Davies & Doroshow, 1986; Wojnowski *et al.*, 2005; Deng & Wojnowski, 2007). An alternative hypothesis implicates a role for topoisomerase II β , present in cardiomyocytes, in doxorubicin-induced myocyte injury (Zhang *et al.*, 2012) (Figure 1.1).

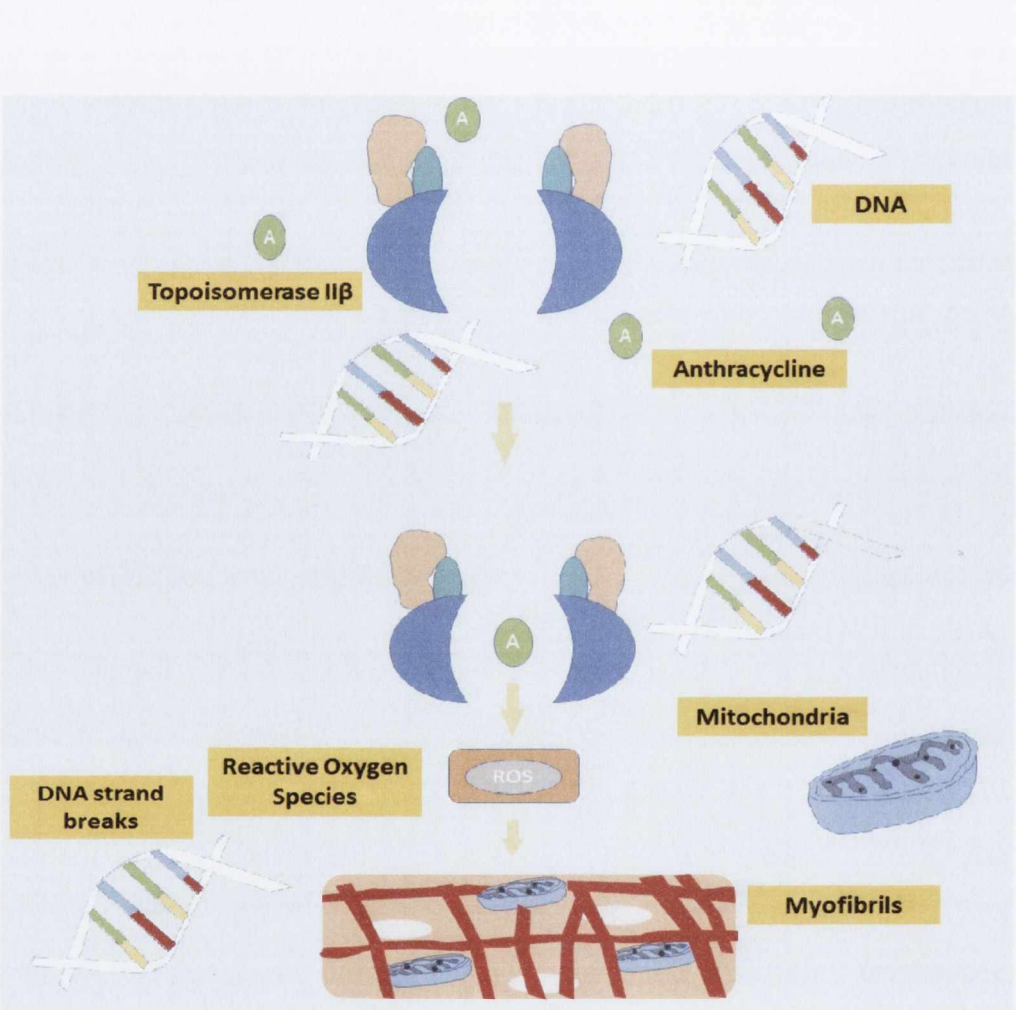


Figure 1.1 Proposed mechanism of anthracycline-mediated cardiac injury. Anthracycline agents alter the activity of topoisomerase II β leading to double-strand breaks in deoxyribonucleic acid (DNA), increased levels of reactive oxygen species and often resulting in mitochondrial dysfunction and significant damage in myofibrils, with vacuolisation.

Extracts from (Vejpongsa & Yeh, 2014).

These factors are linked with a number of different molecular mechanisms which may mediate cardiac injury, potentially resulting in apoptosis or necrosis of cardiomyocytes. The cardiotoxic effects of reactive oxygen species, one of the most commonly recognised mechanisms, can arise following the metabolism of doxorubicin in mitochondrial respiratory complex I and from formation of doxorubicin-iron complexes. This can lead to the generation of free radicals which most likely promotes oxidant stress. Cardiomyocytes are highly susceptible to this type of stress on account of their reliance on oxidative substrate metabolism and the high number of mitochondria located within each cardiomyocyte (Volkova & Russell, 2011). Variants in NAD(P)H oxidase and interference with topoisomerase II β have also been associated with the generation of reactive oxygen species which through the activation of p38 MAPK, caspases 3 and 9 and the release of cytochrome C from the mitochondria into the cytosol have promoted apoptosis of cardiomyocytes (Wojnowski *et al.*, 2005; Volkova & Russell, 2011; Zhang *et al.*, 2012). Additionally, interference with topoisomerase II β has been linked with breaks in DNA double strands and in the transcriptome, which may damage the mitochondria and lead to cardiomyocyte cell death.

Cardiac injury mediated by cardiotoxic chemotherapeutic agents is thought to potentially culminate in a number of pathophysiological modifications in the heart (Ky *et al.*, 2014; Putt *et al.*, 2015) (Figure 1.2). Chemotherapy-mediated cardiac injury is widely regarded to give rise to cardiac myocyte cell death, characterised by a loss of cardiac troponin as well as cardiac remodelling, which may involve activation of neurohormonal pathways such as the B-type natriuretic peptide (BNP) pathway (Cil *et al.*, 2009) and the renin-angiotensin-aldosterone system (RAAS) (Akpek *et al.*, 2015). The BNP pathway is upregulated following ventricular muscle stretch (Kim & Januzzi, 2011), which is often associated with ventricular dysfunction. This can in turn cause the upregulation of the RAAS which is recognised for its role in myocardial remodelling (Solomon & Pfeffer, 2003), including fibrosis which can be associated with galectin-3 (Brilla, 2000). These effects may potentiate the original injury and may give rise to increased risk of more significant cardiac impairment (Hall *et al.*, 2000).

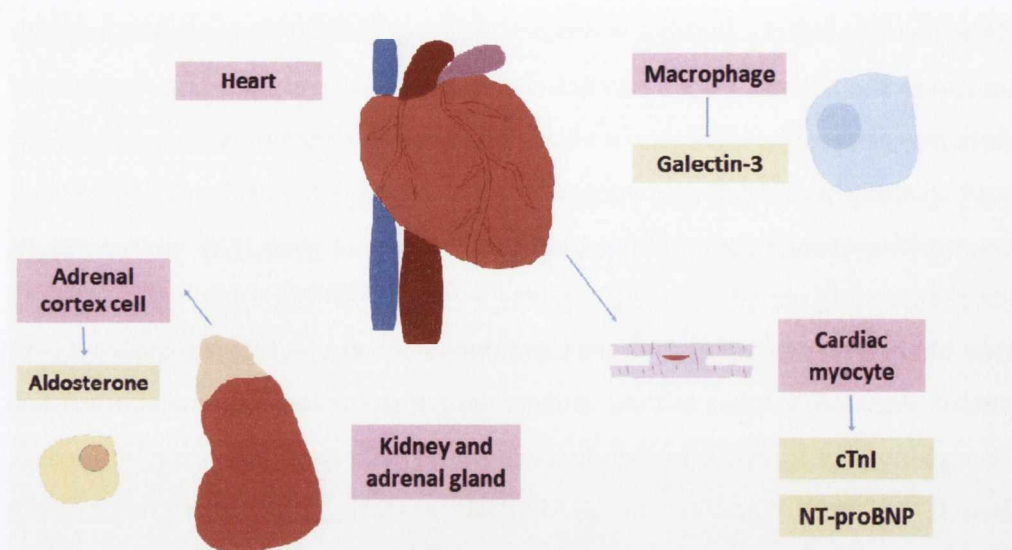


Figure 1.2 Biomarkers potentially associated with cardiac injury. cTnI, cardiac troponin-I; NT-proBNP, amino-terminal pro-B-type natriuretic peptide; galectin-3 and aldosterone are all biomarkers which may be associated with pathophysiological modifications in the heart following injury.

Extracts from (Buglioni & Burnett, 2015).

1.7.1 Detection and monitoring of cardiac injury

Cardiac injury is acknowledged as one of the most toxic sequelae to arise following chemotherapy treatment. Despite the need to detect the earliest indications of cardiac injury, current monitoring protocols for chemotherapy-mediated cardiotoxicity commonly fail to achieve optimal efficacy in detecting such effects in practice (Chavez-MacGregor *et al.*, 2015).

The gold-standard procedure for detecting cardiotoxicity is endomyocardial biopsy, however it is not routinely utilised owing to the potential risks and invasiveness of the procedure. Echocardiography (ECHO) and electrocardiography (ECG) are recognised as a standard means of assessing cardiac function (Curigliano *et al.*, 2012), with decreases in LVEF commonly used to determine cardiotoxicity. However, there are no universal recommendations to guide assessment protocols in the detection of cardiac injury using ECHO or ECG. In addition, ECHO is known to have limited sensitivity in

detecting subtle subclinical cardiac changes (McKillop *et al.*, 1983), which may be indicative of underlying pathological processes in the heart. LVEF decreases are not regarded as the most sensitive or specific means of detecting cardiac injury in patients receiving doxorubicin treatment, as observed in the assessment of congestive heart failure (CHF) (Swain *et al.*, 2003), where significant decreases in LVEF failed to disseminate between patients who developed CHF and those who did not.

1.7.2 High sensitivity cardiac troponin-I in the detection of cardiac myocyte injury

Cardiac troponin I and T (cTnI, cTnT) are both sensitive biomarkers of myocardial cell damage. Highly sensitive assays for cTnI (hs-cTnI) and cTnT (hs-cTnT), have recently been developed with a limit of detection (LOD) of 1.2 ng/L and 5 ng/L for hs-cTnI and hs-cTnT respectively. Although there is no agreed nomenclature, the high sensitive assays are generally designated hs-cTnI and hs-cTnT. cTnI is a 23 kDa subunit of the troponin complex comprised of a distinct 209 amino acid sequence which in association with cardiac troponins-C, T and tropomyosin facilitates muscle contraction (Leszyk *et al.*, 1988). cTnI egresses from the myocyte following myocyte injury (Figure 1.1). On account of the explicit location of cTnI in cardiac myocytes which confers specificity for cardiac muscle, it has been endorsed as the biomarker of choice for the detection of cardiac injury, including in the diagnosis of acute coronary syndromes (ACS) (Thygesen *et al.*, 2012).

cTnI is routinely measured using hs-cTnI immunoassays as defined by Fred Apple in a scorecard designation (Apple & Collinson, 2012). The advent of high sensitivity assays has greatly improved the diagnostic and prognostic potential of this biomarker by virtue of the opportunity to measure substantially lower concentrations of cTnI not previously identifiable with their conventional cTnI immunoassay counterparts, whilst also providing the opportunity to determine gender-specific 99th percentiles for both males and females (Apple, 2009; Apple & Collinson, 2012). Additionally, these assays offer the potential to allow for

more reliable serial assessment of cTnI levels (Jarolim, 2015) and the stratification of two gender-specific cut-offs for males and females.

A comprehensive body of evidence supports a new role for hs-cTnI in the detection of myocardial injury mediated by chemotherapy treatment, most notably by anthracycline agents (Colombo *et al.*, 2014). cTnI concentration has been shown to rise significantly during the course of chemotherapy (Ky *et al.*, 2014). Early studies demonstrated significant correlations between cTnI and the extent of LVEF decrease following high dose chemotherapy (Cardinale *et al.*, 2000; Cardinale, 2002) and has been shown to be significantly associated with subsequent cardiac injury (Cardinale *et al.*, 2004; Sawaya *et al.*, 2012; Ky *et al.*, 2014). Based on these findings, the measurement of cTnI may be of significant potential in the early detection of cardiac injury mediated by chemotherapeutic agents (Curigliano *et al.*, 2012).

While cTnI has demonstrated considerable utility in the specific and sensitive diagnosis of cardiac injury, it does not provide indications regarding the mechanism behind such observations (Sherwood & Kristin Newby, 2014). In addition, sepsis has been shown to potentially mediate increases in cTnI (Ammann *et al.*, 2001). While glomerular filtration rate (GFR) has been speculated to be associated with cTnI, particularly in renal impairment which may potentially result in increased cTnI levels independent of cardiac injury, a recent study failed to demonstrate a strong association between both measures (Bjurman *et al.*, 2015).

1.7.3 Aldosterone and its role in cardiac injury

The association of the RAAS with cardiac injury has prompted the investigation of aldosterone in the detection of such damage (Figure 1.2). Aldosterone is a mineralocorticoid hormone produced in the zona glomerulosa of the adrenal gland, in a region where synthesis and secretion is controlled in a multifactorial manner by circulating levels of angiotensin, sodium and potassium (Kufe *et al.*, 2003). Aldosterone is recognised as a key component of the RAAS with crucial roles in the regulation of blood pressure and cardiac output. Aldosterone promotes

the reabsorption of sodium ions following activation of cytosolic mineralocorticoid receptors in the distal renal tubule and also in the gut while also prompting the secretion of potassium and hydrogen ions (Cartledge & Lawson, 2000; Kufe *et al.*, 2003). In general, circulating levels are considered to be low in normal normotensive individuals (Cartledge & Lawson, 2000).

Aldosterone measurement has clinical utility in the diagnosis of patients with potential secondary causes of hypertension including, primary aldosteronism and other conditions characterised by abnormal levels of aldosterone (White, 1994). More recently, LC-MS/MS has emerged as a potentially superior, robust technique to routinely quantify aldosterone, by virtue of the demonstrated sensitivity across the clinically reportable range and analyte specificity (see section 1.5).

As outlined earlier (see section 1.7), the RAAS has demonstrated critical roles in cardiac remodelling. In particular, aldosterone is recognised to be directly involved in such cases, with animal studies demonstrating the influential role of aldosterone in cardiac endothelial cell proliferation (Gravez *et al.*, 2015), myofibroblast activation (Stockand & Meszaros, 2003) and cardiac fibrosis (Brilla & Weber, 1992). By virtue of its roles, aldosterone has been implicated in many cardiac pathologies including hypertension and HF, where treatment with spironolactone, an aldosterone antagonist, mediated considerable improvements in symptoms and dramatically reduced mortality by 30% (Pitt *et al.*, 1999).

Recent evidence suggests that aldosterone-mediated cardiac injury is dependent on galectin-3 as demonstrated in an experimental study of vascular fibrosis (Calvier *et al.*, 2013). Treatment with the modified citrus pectin, an established inhibitor of galectin-3 prevented hypertrophy and cardiac dysfunction in aldosterone-salt treated rats (Calvier *et al.*, 2015). When investigated more closely in wildtype (WT) and gal-3 knockout (KO) mice, aldosterone treatment was shown to only induce expression of galectin-3 messenger ribonucleic acid (mRNA) and protein in WT mice, which ultimately resulted in increased cardiac collagen I and III expression, indicative of cardiac fibrosis and remodelling. Such

evidence highlights the intricate association and necessary contribution of galectin-3 to aldosterone-mediated cardiac injury and remodelling.

A growing body of evidence has affiliated aldosterone and the RAAS with chemotherapy-mediated cardiotoxicity, with numerous studies postulating a role for aldosterone in evidence of such cardiac injury. The recognised association between aldosterone and cardiac fibrosis (He & Anderson, 2013) has promoted its evaluation for the identification of cardiac injury following anthracycline chemotherapy (Garrone *et al.*, 2011). Furthermore, decreased levels of aldosterone and reduced levels of activity have conferred a degree of protection against cardiac injury mediated by chemotherapeutic agents. Okumura demonstrated increased cardiac angiotensin converting enzyme (ACE) levels in an animal model following anthracycline treatment, with subsequent improvement in cardiac function after the introduction of ACE inhibitors (Okumura *et al.*, 2002). A study by Akpek and colleagues (Akpek *et al.*, 2015) demonstrated that treatment with spironolactone, an aldosterone antagonist, resulted in preserved LVEF in breast cancer patients treated with anthracycline agents, in contrast to patients demonstrating LVEF decreases of greater than 10% after treatment exclusively with anthracyclines.

These findings implicate aldosterone in the development of anthracycline-mediated cardiotoxicity and have provided sufficient basis for its assessment in the detection of chemotherapy-mediated cardiac injury (Garrone *et al.*, 2011). Additionally, where aldosterone is implicated in cardiac injury, quantification of aldosterone may also demonstrate a means of assessing efficacy when treating patients with cardioprotective agents designed to modulate RAAS.

1.7.4 Galectin-3 and its role in cardiac remodelling

Galectin-3, originally termed Mac-2 (Ho & Springer, 1982) is a 30 kDa member of the beta-galactoside-binding protein family (Barondes *et al.*, 1994) which has been the recent focus of investigation in the detection of cardiac injury, by virtue of its role in cardiac remodelling and fibrosis. It is comprised of two domains, a

collagen-like N-terminal domain necessary for extracellular secretion and a C-terminal carbohydrate recognition domain which facilitates lectin-binding (Dumic *et al.*, 2006). While predominantly released from newly differentiated macrophages (Liu *et al.*, 1995) (Figure 1.2), its expression has previously been noted in the nucleus, cytoplasm and on the cell surface of many other cells including mast cells, eosinophils and neutrophils (de Boer *et al.*, 2010). Experimental research has shown that galectin-3 is expressed at high levels in numerous organs including in the lungs, colon and stomach, which contrasts with organs such as the kidneys and heart which are documented to have low levels of expression (Kim *et al.*, 2007). On account of its extensive expression profile, galectin-3 is characterised by an expansive functional profile with recognised regulatory roles in inflammation, immunity and cancer (de Boer *et al.*, 2010). In particular, the significant impact of galectin-3 in cardiac fibrosis and remodelling has promoted its utility as a biomarker in cardiac pathologies with a potential role in the detection of chemotherapy-mediated cardiotoxicity.

There is a compelling body of evidence which demonstrates the association between galectin-3 and cardiac injury on account of its role in cardiac remodelling and fibrosis, particularly in cases of HF (Sharma *et al.*, 2004; Yu *et al.*, 2013). On account of the potential role of cardiac fibrosis in heart failure, galectin-3 has been evaluated in this setting, demonstrating significantly elevated levels in patients diagnosed with acute HF (van Kimmenade *et al.*, 2006), a finding also confirmed in patients diagnosed with chronic HF (Lok *et al.*, 2010). Furthermore, elevated galectin-3 levels have been associated with an increased risk of incident HF (Ho *et al.*, 2012), which when taken together implicate galectin-3 in the development of HF where cardiac remodelling is a significant feature (de Boer *et al.*, 2009).

Galectin-3 is recognised to be a crucial component of aldosterone-mediated cardiac injury, with knock-out or inhibition recognised to prevent cardiac remodelling induced by aldosterone (Calvier *et al.*, 2013; Calvier *et al.*, 2015). As some chemotherapeutic agents may confer significant risk of hypertension (Grossman *et al.*, 2015), the association between both biomarkers will be crucial to investigating the possibility of cardiac injury induced by aldosterone-mediated cardiac remodelling. As such, any association between both biomarkers will likely

confer additional risk for cardiac fibrosis and remodelling owing to their intricate links.

Based on these findings and given the recognition of HF as an acknowledged side effect of chemotherapy (Yeh & Bickford, 2009), galectin-3 may be a useful biomarker to facilitate detection of cardiac remodelling and HF. More importantly, the cardiac injury mediated by chemotherapeutic agents may resemble aspects of the cardiac injuries which are mediated by myocardial infarction (MI) including cardiac myocyte damage demonstrated by increased cTnI levels (Cardinale *et al.*, 2000). Such mechanistic links are important given the recognition that post-injury myocardial remodelling can arise following MI, to which galectin-3 plays a critical role (Meijers *et al.*, 2015). It is reasonable to suggest such remodelling could also occur following chemotherapy-mediated cardiac injury, thus promoting its assessment in patients treated with potentially cardiotoxic chemotherapy.

Galectin-3 has been shown to demonstrate a significant association with ventricular dysfunction, a known toxic sequelae of cardiotoxic chemotherapy (Yoon *et al.*, 2010). Patients with MI who exhibit elevated galectin-3 levels (>17.8 ng/mL) at baseline were found to have lower LVEF values four months post-MI when compared with those with normal galectin-3 measures (<17.8 ng/mL) (van der Velde *et al.*, 2015). Given the demonstrated role in cardiac remodelling, this finding links galectin-3 with the establishment of ventricular dysfunction. In an assessment of human myocardial biopsies by Sharma and colleagues (Sharma *et al.*, 2004) where known cardiac hypertrophy was associated with decreases in ejection fraction (EF), elevated levels of galectin-3 mRNA expression were demonstrated when compared with compensated cases. Based on the findings from such studies there is a suggestion that such increases in galectin-3 are common to many forms of left ventricular (LV) dysfunction (de Boer *et al.*, 2010) and may in fact be a suitable index of such injury. Patients who receive cardiotoxic chemotherapy, particularly anthracycline agents are acknowledged to be at risk of LV dysfunction (Cardinale *et al.*, 2015) and this biomarker may demonstrate a novel means of detecting evidence of such ventricular changes.

Galectin-3, has also been linked with superoxide radicals (Liu *et al.*, 1995). Previously, evidence has been published which suggests it prompts superoxide production by neutrophils (Yamaoka *et al.*, 1995), which are recruited to the heart in episodes of cardiac stress, by phagocytic macrophages (Hulsmans *et al.*, 2015), known to release galectin-3. As superoxide radicals have previously been associated with anthracycline-mediated cardiotoxicity (Minotti *et al.*, 2004), galectin-3 may potentially be an influential factor in this relationship, thus promoting its assessment in patients treated with potentially cardiotoxic chemotherapeutic agents.

The prognostic role of galectin-3 has also been extensively characterised in recent years particularly in HF (Lok *et al.*, 2010) and recently advocated for their additive prognostic value in association with natriuretic peptides in HF (Yancy *et al.*, 2013).

1.7.5 Amino-terminal pro-B-type natriuretic peptide and its role in cardiac injury

The B-type natriuretic peptides (BNP and NT-proBNP) are regarded as optimal diagnostic and prognostic biomarkers in heart failure. NT-proBNP has emerged as a biomarker of considerable interest in the detection of chemotherapy-mediated cardiac injury (Figure 1.1). NT-proBNP is a biologically inactive, 76 amino acid cleavage peptide of the prohormone proBNP which is transcribed from the BNP gene in cardiac myocytes (Kim & Januzzi, 2011) (Figure 1.3). It is released primarily from ventricular cardiac myocytes following myocardial stress induced by pressure overload or cardiac stretch in a sign of neuroendocrine system activation (Clerico *et al.*, 2006). NT-proBNP is secreted into the circulation in equimolar proportions to BNP, the biologically active cleavage fragment of proBNP which serves to promote vasodilation and diuresis among other functions (Hall, 2004). The half-life of NT-proBNP has been reported to be between 60 and 120 minutes with clearance predominantly mediated by the kidneys (Palmer *et al.*, 2009). Albeit to a smaller degree, the liver and skeletal tissues have also been documented to play a role in NT-proBNP clearance (Palmer *et al.*, 2009). Owing to its longer half-life (60 minutes) when compared with BNP (20 minutes),

circulating levels of NT-proBNP have been shown to be generally greater than BNP. In healthy individuals, levels of NT-proBNP are recognised to be higher in females when compared with males (Campbell *et al.*, 2000) and are noted to rise with increasing age (Raymond *et al.*, 2003). NT-proBNP is measured in blood by immunoassay, with evidence which suggests superior sample stability when compared with BNP. NT-proBNP has previously received US Food and Drug Administration (FDA) clearance for the assessment of individuals with suspected CHF owing to its release kinetics and as such has warranted much interest in the investigation of chemotherapy-mediated cardiac injury.

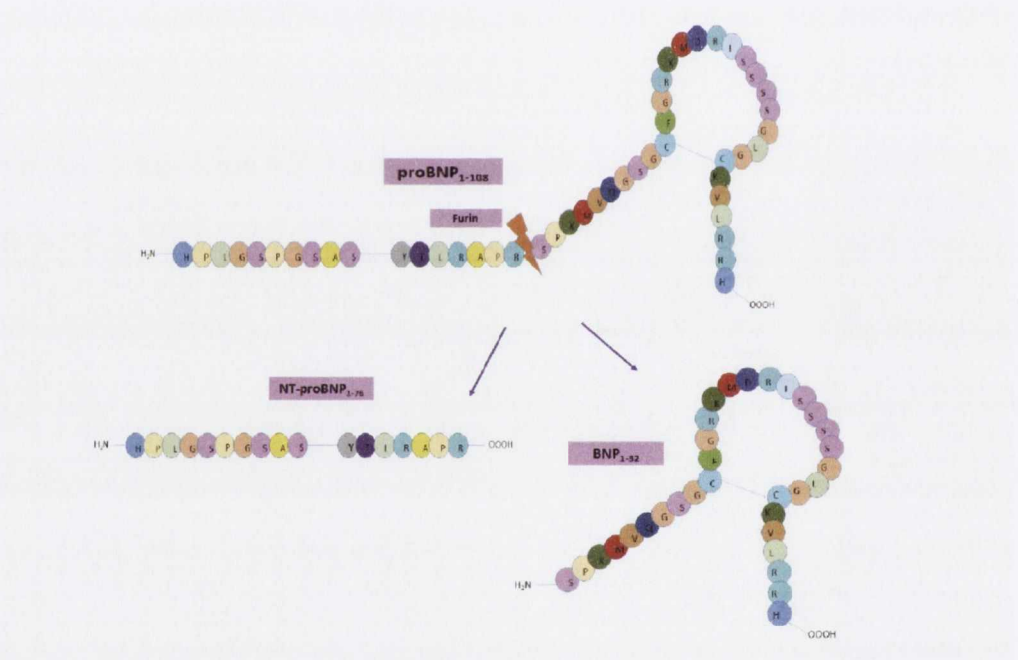


Figure 1.3 Cleavage of NT-proBNP from the prohormone proBNP₁₋₁₀₈. proBNP is enzymatically cleaved by furin into NT-proBNP and BNP.

Extracts from (Kim & Januzzi, 2011)

The prevailing myocardial stress mechanism which prompts the release of NT-proBNP has promoted its use as a biomarker associated with ventricular dysfunction. Increases in NT-proBNP concentration have been strongly linked in a large hospitalised patient cohort with decreasing LVEF (Bay *et al.*, 2003), known to be suggestive of ventricular dysfunction. Patients in this study who presented with an LVEF below or equal to 40% had significantly higher NT-proBNP levels compared with patients with an LVEF above 40% and 50% respectively, which indicates that the severity of the dysfunction can be accompanied by a marked increase in NT-proBNP concentration. Similarly high levels of NT-proBNP have been reported in patients investigated for heart failure who also presented with decreased LVEF (Gustafsson *et al.*, 2005).

By virtue of its diagnostic efficacy, NT-proBNP has also provided a potential means of detecting ventricular dysfunction, often characterised as decreased LVEF (Curigliano *et al.*, 2012) in chemotherapy-mediated cardiotoxicity, induced by agents which include the anthracyclines (Yoon *et al.*, 2010) particularly in patients with mildly decreased LVEF (Mueller *et al.*, 2004). This suggests that NT-proBNP performs optimally in the identification of early signs of ventricular dysfunction which justifies its investigation for the early detection of anthracycline-mediated decreases in LVEF. In a study by Cil and colleagues, elevated levels of NT-proBNP were linked with a decreased LVEF in asymptomatic patients following doxorubicin treatment (Cil *et al.*, 2009), which demonstrated the capability of NT-proBNP to identify patients with ventricular dysfunction prior to symptomatic presentation,, a finding supported in other studies (Romano *et al.*, 2011). Such findings demonstrate the potential for this biomarker to facilitate early detection of asymptomatic ventricular dysfunction. NT-proBNP has demonstrated superior potential when compared with echocardiographic-based evaluation of LVEF for the detection of cardiac injury in patients treated with an anthracycline-based chemotherapeutic regimen. This may be due to the fact that NT-proBNP is recognised as a more efficient marker of cardiac myocyte stress when compared with LVEF (deFilippi & Christenson, 2009). Furthermore, patients who demonstrated a persistently elevated NT-proBNP measurement immediately following infusion of the chemotherapeutic

agent were shown to present with considerable decreases in LVEF following completion of chemotherapy regimen (Sandri *et al.*, 2005). A significant rise in NT-proBNP concentration was identified prior to decreases in LVEF in breast cancer patients treated with anthracycline-based chemotherapeutic regimen (De Iuliis *et al.*, 2015; *in press*). NT-proBNP has provided compelling indications from these studies for its use in the earlier detection of cardiac injury.

NT-proBNP plays a critical role as a biomarker in the routine diagnosis (Lainchbury *et al.*, 2003) and rule-out of heart failure (Don-Wauchope & McKelvie, 2015), where detection of significantly elevated levels of NT-proBNP in patients have been demonstrated to greatly improve diagnostic accuracy (Moe *et al.*, 2007). The clinical utility of this biomarker in the heart failure setting has advanced beyond a purely diagnostic role and has been deemed useful for the determination of the severity of heart failure cases based on the natriuretic peptide levels (Maisel *et al.*, 2008). This finding lends itself to the ability to risk stratify patients based on the concentration of the biomarker measured.

In patients treated with anthracycline agents, heart failure has been recognised as a potentially fatal side effect of treatment (Von Hoff *et al.*, 1979), which warrants early detection and intervention to ameliorate or restore appropriate levels of cardiac function. A recent study of BNP measured in patients during anthracycline-based chemotherapy treatment demonstrated elevated BNP levels in patients who were subsequently admitted for CHF (Skovgaard *et al.*, 2014). While it was noted that BNP and not NT-proBNP was assessed by Skovgaard and colleagues, both biomarkers are often assessed interchangeably owing to similar diagnostic performance in the detection of heart failure (Clerico *et al.*, 2007).

The prognostic role of NT-proBNP has also been extensively reviewed. In patients diagnosed with acute heart failure combined with ventricular dysfunction, persistently elevated NT-proBNP levels were strongly associated with mortality, with a better prognosis when a greater reduction in NT-proBNP measures was detected (greater than 30% reduction) (Bayés-Genís *et al.*, 2005). These findings propose a means of prognostic assessment of patients receiving cardiotoxic

anthracycline chemotherapy, where elevated measures may indicate a worse prognosis.

The principal clearance mechanism of NT-proBNP from the kidneys (Palmer *et al.*, 2009), impaired renal function has been considered to pose a significant caveat to the interpretation of elevated measures which would otherwise be indicative of cardiac injury (deFilippi & Christenson, 2009). This is in addition to the concept that cardiac and renal function are inexplicably intertwined. In cases where estimated GFR (eGFR) decreases below 60 ml/min/1.73m², the level of NT-proBNP has been shown to dramatically increase (Jafri *et al.*, 2013). However, in a study of over 200 ambulatory patients with chronic kidney disease, NT-proBNP remained an indicator of cardiac injury, classified as ischemic heart disease and hypertrophy following adjustment for GFR which indicates that its measurement should remain important to clinical assessment (deFilippi *et al.*, 2005). Careful interpretation of raised B-type natriuretic peptides in the presence of decreased GFR is advised.

1.8 Renal injury during chemotherapy treatment

The kidneys perform key roles in the homeostasis, including the maintenance of body-fluid balance, hormone production and excretion of metabolic and therapeutic waste products, including chemotherapeutic agents. Renal injury is a significant complication in patients receiving chemotherapy treatment which commonly presents acute kidney injury (AKI), acute and chronic kidney disease (AKD and CKD respectively) or renal failure (RF) (Perazella & Moeckel, 2010). Cisplatin and to a lesser extent carboplatin are recognised as potentially nephrotoxic agents, recognised to cause kidney disease (Ardizzoni *et al.*, 2007). The prevalence of renal injury in oncology is significant, with an extensive study of 4,600 oncology patients demonstrating evidence of mild-to-moderate renal impairment in over 50% of study participants (Launay-Vacher *et al.*, 2007). Additionally, AKI has been identified in almost 50% of terminal cancer patients (Lacava *et al.*, 2015) highlighting the need to detect evidence of impairment using the most sensitive diagnostic methods.

1.8.1 Monitoring and detection of kidney injury

Renal function is evaluated in oncology using biomarkers which are measured for the examination of GFR or creatinine clearance (CrCl). GFR is widely regarded as an effective and reliable indicator of kidney function and is assessed either by indirect measurement or using estimating formulae (Foundation, 2002). CrCl can also be measured or estimated to provide an alternative assessment to GFR. However in spite of the many methods available, the paucity of a globally accepted and routinely applicable technique has allowed for discrepancy in the manner by which renal function is examined.

The indirect measurement of GFR (measured GFR, mGFR) is regarded as the gold-standard assessment method to accurately evaluate GFR and in turn renal function (Stevens *et al.*, 2006; Levey *et al.*, 2014b). Assessed by the clearance of an exogenous biomarker such as inulin or chromium EDTA (^{51}Cr -EDTA) from the kidneys, mGFR is the benchmark technique for evaluating GFR in oncology (Michels *et al.*, 2010; Ainsworth *et al.*, 2012; Chew-Harris *et al.*, 2015). However, mGFR is not routinely performed in oncology patients by virtue of its expensive, labour-intensive and time consuming nature (Hsu & Bansal, 2011). On account of the associated issues, more feasible alternatives using estimates of GFR have recently been sought which achieve similar levels of accuracy (Lauritsen *et al.*, 2014; Chew-Harris *et al.*, 2015).

Estimation of GFR is routinely performed in oncology using endogenous serum biomarkers which are employed in formulae to estimate GFR (Stevens *et al.*, 2006; Levey *et al.*, 2015). Creatinine and cystatin C are the two major endogenous biomarkers used in such evaluations of renal function. Creatinine is a 113 Da breakdown product of creatine in muscle which is generated at a relatively constant rate (Heymsfield *et al.*, 1983). It is a physiologically inert molecule, freely filtered by the glomerulus and not bound to plasma proteins (Perrone *et al.*, 1992). Creatinine is typically measured using an enzymatic assay or kinetic Jaffe method and has been standardised to a National Institute of Standards and Technology (NIST) international reference material which is traced to an isotope dilution mass spectrometry method (NIST standard reference material (SRM)

967). Creatinine serves as the biomarker of choice for the routine estimation of GFR using formulae including the Modified Diet in Renal Disease (MDRD) and more recently in the Chronic Kidney Disease-Epidemiology collaboration (CKD-EPI) formulae. In addition, creatinine demonstrates utility in the measurement of CrCl and in its estimation using the Cockcroft-Gault (CG) formula. Changes in creatinine concentration are monitored for evidence of AKI (Levey *et al.*, 2015), characterised as a sudden decline in kidney function and defined by a significant increase in serum creatinine concentration or reduction in urine output over 48 hours as classified most recently by the Acute Kidney Injury Network (AKIN) (Lopes & Jorge, 2013).

Creatinine is affected by patient characteristics and analytical variability. Parameters such as age, gender (Hsu *et al.*, 2002), muscle mass (Baxmann *et al.*, 2008), dietary protein levels (Perrone *et al.*, 1992) and medications such as trimethoprim (Berg *et al.*, 1989) can influence its concentration independent of changes in renal function. Creatinine is also known to be secreted by renal proximal tubules and can potentially overestimate CrCl in comparison to measured GFR by as much as 40% (Shemesh *et al.*, 1985; Perrone *et al.*, 1992). Analytical issues including non-creatinine chromogens which can interfere with creatinine measurement. In addition it has been noted that renal function is required to deteriorate by 50% before any appreciable increase in serum creatinine levels is observed (Renkin & Robinson, 1974; Apple *et al.*, 1989), which significantly limits the sensitivity of the biomarker to detect early indications of losses in renal function. In view of these issues, the overwhelming opinion in literature advises against sole reliance on the assessment of creatinine for the general assessment of renal function (Levey *et al.*, 2015).

The limitations which influence creatinine measurement have prompted the evaluation of cystatin C. Cystatin C is a 13 kDa basic protein of 120 amino acids produced by virtually all nucleated cells at a constant rate which primarily functions as a cysteine proteinase inhibitor. It is freely filtered by the glomerulus with subsequent endocytosis and metabolism by the proximal renal tubule. Cystatin C has a shorter plasma half-life (90 minutes) when compared with creatinine and has been shown to more rapidly demonstrate alterations in renal

function (Sjöström *et al.*, 2005; Lima *et al.*, 2014), which is postulated to confer improved sensitivity in detecting small changes in eGFR (Sjöström *et al.*, 2005). There is additional benefit in that cystatin C measurements are less affected by muscle mass (Vinge *et al.*, 1999) and protein intake (Tangri *et al.*, 2011). Cystatin C is now measured using immunoturbidimetric and nephelometric assays which were recently standardised to a European reference material (ERM DA-471/IFCC). This biomarker has demonstrated considerable utility in newly promoted cystatin C-based CKD-EPI eGFR formulae, with evidence which suggests that cystatin C performance at higher GFR measures is significantly better than creatinine (Stevens *et al.*, 2006).

However, there are known limitations to the assessment of cystatin C which include impaired thyroid function (Manetti *et al.*, 2005) and corticosteroid use (Risch *et al.*, 2001). In addition, many studies have demonstrated increased levels of cystatin C in various malignancies where impaired renal function was not noted (Naumnik *et al.*, 2009; Tumminello *et al.*, 2009; Chen *et al.*, 2011).

1.8.2 Estimating formulae used to assess kidney function

There is an abundance of formulae available to routinely estimate GFR in practice. The original MDRD formula which has been re-expressed following the standardisation of creatinine (Levey *et al.*, 2007), and is one of the most commonly applied formulae (see Appendix I for formula detail). However, studies have shown that MDRD significantly underestimates mGFR particularly at eGFR values above 60 ml/min/1.73m², including in oncology patients (Stevens *et al.*, 2007; Dooley *et al.*, 2013), which may lead to the inappropriate classification of more significant renal impairment than present when compared with more accurate techniques (Matsushita *et al.*, 2012). For these reasons, eGFR measurements above 60 ml/min/1.73m² are generally reported as >60 ml/min/1.73m², and subtle changes in kidney function can be missed. Gender and ethnicity are accounted for in estimating formulae but, careful interpretation is warranted as estimates of GFR are confounded by other factors including body mass index (BMI), pregnancy, diabetes and is recognised to decrease with age.

The shortcomings of eGFR-MDRD prompted the development of the CKD-EPI formula which was designed to improve the accuracy of assessing kidney function above 60 ml/min/1.73m² (see Appendix I). The three CKD-EPI formulae derived using either creatinine (SCr), cystatin C (CysC) or a combination of both biomarkers (SCr/CysC) have been shown to more accurately predict decreases in GFR when compared with CG (Michels *et al.*, 2010; Chew-Harris *et al.*, 2015), demonstrating a greater proportion of eGFR measures within 20% of the mGFR calculated value when compared with CG. CKD-EPI SCr is also recognised to demonstrate superior performance to MDRD (Levey *et al.*, 2014b), with mean eGFR measures which were found to be more closely aligned with mGFR (Dias *et al.*, 2013; Dooley *et al.*, 2013). When directly compared with MDRD, CKD-EPI SCr was shown to contribute to the classification of fewer patients with CKD (Matsushita *et al.*, 2012). By virtue of the substantial body of evidence demonstrating improved accuracy in estimating GFR, the CKD-EPI SCr-based formula has been advocated in the KDIGO guidelines for the evaluation of eGFR (Global & Group., 2013). CKD-EPI CysC has been suggested for general patient use to confirm CKD stage when eGFR as calculated using CKD-EPI SCr is between 45-59 ml/min/1.73m² and where other markers of kidney injury, for example, albuminuria are absent. Recent studies have indicated the combined CKD-EPI SCr/CysC formula outperforms the CKD-EPI SCr and CKD-EPI CysC formulae (Bjork *et al.*, 2015; Meeusen *et al.*, 2015). Despite the evidence for the superiority of CKD-EPI over CG and MDRD (Chew-Harris *et al.*, 2015; Parsh *et al.*, 2015), the latter remain the standard of practice in clinical diagnostics. This may be in part due to the recognition that fewer studies which have investigated CKD-EPI have been performed (Levey *et al.*, 2014a).

Alternatively, the analysis of CrCl, either by measurement or estimation has afforded another means of evaluating kidney function. A drawback to the utility of CrCl is the inconvenient and cumbersome nature of 24-hour urine sample collection which can lead to inaccuracy in assessment. Additionally, as outlined CrCl is often recognised to overestimate GFR due to the tubular secretion of creatinine. The CG formula was designed to circumvent the issues of measured CrCl through the estimation of creatinine clearance (Cockcroft & Gault, 1976)

(see Appendix I). However despite its continual use this formula is associated with inherent inaccuracies including its derivation in a male cohort and more importantly, the mathematical derivation was constructed with a different and unstandardized creatinine method resulting in significant levels of underestimation when compared with mGFR (Michels *et al.*, 2010; Dooley *et al.*, 2013; Chew-Harris *et al.*, 2015). Current standardised creatinine methods have an upper reference range that is on average 26 $\mu\text{mol/L}$ lower than the method that was used for CG calculation. In addition to the poor accuracy demonstrated, CG is influenced by body weight and body mass index (Michels *et al.*, 2010).

Additional tests such as urinalysis and specific biomarkers of renal injury complement the assessment techniques available to facilitate a means of detecting renal injury in oncology patients. However, no method has gained universal accuracy to evaluate renal function. The disadvantages associated with current methods reaffirm the need for a robust and extensively validated technique which can be routinely utilised in oncology patients where the assessment of kidney function has implications in the domain of chemotherapeutic drug dosing.

1.8.3 The use of renal estimating formula in the calculation of chemotherapeutic agent doses

One of the most pertinent examples in oncology where the assessment of renal function is critical is in chemotherapeutic dosing. Most hospital pharmacy departments routinely utilise CG in the Calvert formula to calculate carboplatin doses (Calvert *et al.*, 1989) (see Appendix I). However, as previously outlined, CG is an inherently inaccurate formula. There is a substantial body of evidence which demonstrates significant disagreement between CG-calculated carboplatin doses and doses calculated using eGFR and mGFR formulae. Numerous studies have shown significant levels of carboplatin underdosing using CG, with two studies which reported that over 30% and 40% of CG-calculated doses were at least 20% lower than the mGFR-calculated carboplatin dose (Dooley *et al.*, 2013; Cathomas *et al.*, 2014; Bertelli *et al.*, 2015; Chew-Harris *et al.*, 2015). It is acknowledged that expression of GFR measurements is relative to an average

surface area of 1.73m^2 to account for the relationship between renal function, weight and height. However, in the case of carboplatin dosing, body surface area (BSA) indexing is not a factor in the calculation of chemotherapeutic doses and its removal has been suggested in such cases (Levey *et al.*, 2015). The removal of BSA indexing has been shown to contribute to a 10% increase in dosing concordance for carboplatin using CKD-EPI, which may have significant therapeutic benefits.

1.8.4 Neutrophil-gelatinase associated lipocalin and the detection of kidney injury

Early clinical and biochemical indicators of AKI are lacking. There is no troponin-like biomarker for AKI. In recent years, neutrophil gelatinase associated lipocalin (NGAL) has demonstrated potential in the detection of AKI (Nickolas *et al.*, 2008; Soto *et al.*, 2013) which may be a beneficial application in oncology. NGAL is a 25 kDa member of the lipocalin family typically expressed in cells including neutrophils and macrophages at low concentrations. Following injury, concentrations of NGAL can increase significantly. NGAL encompasses an extensive array of roles which include protection from bacterial infection and regulation of oxidative stress (Mori & Nakao, 2007; Chakraborty *et al.*, 2012). More recently, NGAL has also been linked with tumour growth in cancer (Candido *et al.*, 2014), which has prompted a new wave of research in oncology.

In a Sprague Dawley mouse model of ischemia, upregulation of NGAL expression observed three hours post-injury in the proximal tubules, complemented the rapid detection of NGAL in the urine of mouse and rat-models of acute renal failure (Mishra *et al.*, 2003). Furthermore, in rats treated with lipopolysaccharide to induce AKI, increased levels of NGAL mRNA expression and peak levels of urinary NGAL were detected in the first 12 hours-post LPS treatment which coincided with evidence of AKI (Han *et al.*, 2012). Elevated levels of NGAL have previously been shown to precede AKI in multiple trauma patients (Makris *et al.*, 2009), with significantly elevated NGAL levels also detected in patients with AKI compared with other conditions including CKD and patients with normal renal function (Nickolas *et al.*, 2008). NGAL levels have

also been shown to increase with severity of AKI (Soto *et al.*, 2013). Furthermore, increased NGAL expression has preceded increases in creatinine levels (Mishra *et al.*, 2003; Mishra *et al.*, 2005), indicative of an earlier means of detecting renal injury. These findings indicate promise for the reliability and timely nature of NGAL to detect AKI.

The utility of NGAL in oncology populations, known to have generally low neutrophil counts, may be emphasised in these patients in the event of renal impairment, where NGAL levels would be strongly suspected to correlate with release from the renal tubules. AKI is a recognised complication of chemotherapy treatment which is associated with a worse prognosis (Lahoti *et al.*, 2010) and in such cases the evaluation of NGAL may prove beneficial.

1.9 Study objectives

The objectives of this study were to:

- To evaluate the utility of hs-cTnI, galectin-3 and NT-proBNP levels in the detection of subclinical chemotherapy-mediated cardiac injury
- To develop a sensitive and specific LC-MS/MS method for the quantification of aldosterone and to evaluate the suitability of this method to be applied in an oncology cohort
 - Additionally to determine the potential of aldosterone to identify subclinical chemotherapy-mediated cardiac injury
- To evaluate cystatin C and to compare with creatinine for the assessment of kidney function using mathematically derived estimating formulae
- To compare the newly advocated CKD-EPI formula with CG in current chemotherapeutic drug dosing protocols using the Calvert formula
- To evaluate NGAL in the detection of acute kidney injury during chemotherapy treatment

Chapter 2

Methods

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2.1 Assessment of cardiac and renal biomarkers

2.1.1 Sample collection and processing

The cardiac biomarkers hs-cTnI (Abbott Diagnostics, USA), galectin-3 (Abbott Diagnostics, USA), NT-proBNP (Roche Diagnostics GmbH, Germany), aldosterone (Waters, UK) and renal biomarkers cystatin C (Abbott Diagnostics, USA) and creatinine (Abbott Diagnostics, USA) were assessed from a serum sample (collected in gel separator tubes; Sarstedt, Germany), while the renal biomarker NGAL (Abbott Diagnostics, USA) was assessed from a urine sample collected prior to the commencement of each chemotherapeutic cycle. Samples were centrifuged at 3,500 g for 10 minutes (Eppendorf 5810 centrifuge, Eppendorf, Ireland) and frozen at -70°C until analysis.

2.1.2 Immunoassay analysis of cardiac and renal biomarkers

The concentrations of hs-cTnI, galectin-3 and NGAL were assessed using a 2-step immunoassay which utilised a chemiluminescent acridinium-labelled conjugate. Following incubation of the sample with both antibodies, photons of light emitted from the biomarker-conjugate complex were quantified and correlated to the concentration of the biomarker in the sample. The concentration of NT-proBNP was determined by an electrochemiluminescence immunoassay technique. Following antibody-antigen incubation, the application of a voltage to the ruthenium-labelled antibody complexed with NT-proBNP leads to the emission of photons of light, quantifiable to the concentration of NT-proBNP in the sample. Assay characteristics are described in Table 2.1. Aldosterone was assayed using a LC-MS/MS assay which was developed in this study (see Chapter 3).

Cystatin C was assessed using a particle-enhanced turbidimetric immunoassay (PETIA) in which the agglutination of cystatin C to antibody-coated latex particles induced a change in absorbance which was quantifiable to the concentration of cystatin C. This assay was standardized to the European reference material for cystatin C, ERM-DA 471/IFCC. Creatinine was assessed using an enzymatic procedure in which a series of hydrolysis and oxidation steps resulted in a change in the absorbance of the sample which was quantified to yield

the concentration of creatinine. This assay was an isotope dilution mass spectrometry (IDMS)-traceable method to the NIST standard reference material (SRM) 967.

2.1.2.1 Preparation and analysis of samples

Serum and urine samples were thawed, mixed and centrifuged at 3,500 g for 10 minutes for hs-cTnI, NT-proBNP, creatinine, cystatin C and NGAL (Megafuge 1.0 Heraeus Sepatech, Abbott Diagnostics) and at 11,000 g for galectin-3 for five minutes (X-Systems Microfuge Model 3530, Abbott Diagnostics) prior to assessment.

Samples prepared for hs-cTnI, galectin-3 and NGAL analysis were combined in a reaction vessel with the first monoclonal antibody (mouse anti-troponin-I, M3/38 anti-galectin-3 and mouse anti-NGAL respectively). Samples were incubated followed by a wash step and combined with a second monoclonal antibody (human-mouse chimeric anti-troponin, 87B5 anti-galectin-3 and mouse anti-NGAL respectively) which was coated on an acridinium-labelled conjugate. A further incubation period and wash step were executed and biomarker concentrations were determined from the chemiluminescent reaction generated. Serum samples for NT-proBNP analysis were combined with a biotinylated monoclonal NT-proBNP-specific antibody and a monoclonal NT-proBNP-specific antibody labelled with a ruthenium complex. Samples were incubated, combined with streptavidin-coated microparticles and bound to an electrode. A voltage was applied resulting in an electrochemiluminescent reaction from which NT-proBNP was quantified. Serum samples for cystatin C analysis were combined with anti-human cystatin C antibodies and the concentration was determined from the change in the absorbance of the mixture. Serum samples assessed enzymatically for creatinine were hydrolysed to generate creatine, which was in turn hydrolysed to sarcosine. The oxidation of sarcosine resulted in the generation of hydrogen peroxide which consequently lead to the generation of a quinoneimine dye. The change in the absorbance of the mixture was comparable to the concentration of creatinine present in the sample.

Table 2.1 Assay and instrument characteristics.

Name	Assay Type	Analytical CV	LOD	Assay Range	Instrument (Abbott Architect)	External EQA
hs-cTnI	2-step IA	≤ 5.3 % *	1.2 ng/L	5-50,000 ng/L	i2000sr	UK NEQAS †
GAL-3	2-step IA	≤ 3 % **	1.0 ng/mL	4-114 ng/mL	i1000sr	NA
NT-proBNP	2-step IA	≤ 4.6 % ***	5.0 pg/mL	44-35,000 pg/mL	Cobas e411♦	NA†††
Cystatin C	PETIA	≤ 2 % §	0.05 mg/L	0.05–9.78 mg/L	c16000	NA†††
NGAL	2-step IA	≤ 10 % §§	1.0 µg/L	10-1,500 µg/L	i1000sr	NA
Creatinine	ENZ	≤ 2 % §§§	8.8 µmol/L	8.8-3,536 µmol/L	c16000	UK NEQAS††

Analytical CV was evaluated across the following QC ranges: *11-16,444 ng/L; **9-74 ng/mL; ***44-33,606 pg/mL; §0.9-3.98 mg/L; §§ 20-1,200 µg/L; §§§6-577 µmol/L. † UK NEQAS for Cardiac Biomarkers performed routinely, †† UK NEQAS for General Clinical Chemistry performed routinely, ††† UK NEQAS schemes available, but assays not used routinely. ♦Roche cobas e411 analyser. IA, immunoassay; ENZ, enzymatic; NEQAS, National External Quality Assessment Scheme; PETIA, particle-enhanced turbidimetric immunoassay.

Verification of hs-cTnI assay precision according to Clinical and Laboratory Standards Institute (CLSI) guidelines (EP15-A3) and validation of all Abbott Architect i2000sr analysers was performed previously within the Mater Diagnostic laboratory. Assay precision was evaluated in accordance with CLSI guidelines (EP15-A2) for all other biomarkers.

2.1.3 Assessment for sample interference

Samples were examined for hemolysis, icterus and lipemia by means of a spectrophotometric on-instrument assessment during cystatin C analysis (Abbott Architect c16000, Abbott Diagnostics, USA). Manufacturer-recommended rejection limits, verified within the Mater Diagnostic laboratory, were used for assessment and samples beyond acceptable levels were rejected.

2.1.4 Assessment of patient clinical characteristics

C-reactive protein and white blood cell counts were obtained from patient records in cases of elevated hs-cTnI measurements. ECHO results were examined with LVEF and cardiologist ECHO interpretations recorded. Patient weight and BSA details were collected from pharmacy records.

2.1.5 Generation of a test result database

A database was generated for the collation of patient data and biomarker measurements. Patient age at diagnosis, chemotherapy regimen details and cohort assignment were also recorded. A spreadsheet was created to collate chemotherapeutic drug doses in addition to BSA, creatinine and cystatin C data, which were utilised to calculate creatinine clearance and estimated glomerular filtration rates for each cycle of chemotherapy.

2.2 Patient recruitment with disease and treatment characteristics

2.2.1 Recruitment of patients to the study

Ethical approval was attained prior to commencing patient recruitment (Ref: 1/378/1387). Patients due to commence chemotherapy treatment for the first time were selected from the oncology/haematology wards. Patients were recruited following signed consent to participation and assessed during the course of chemotherapy.

2.2.2 General characteristics of the study cohort

Eighty-four patients were recruited to the study, of whom 66 were female (see Table 2.2) and assessed for a median of eight cycles of chemotherapy (IQR: 6-12 cycles). At final assessment, 14 of the 84 patients were deceased.

Table 2.2 Characteristics of the study cohort.

General characteristics		Patient total
Patient number		84
M, F		18, 66
Mean age at enrolment (SD)		55 years (12.5)
M, F		62 (12.1), 53 (12.2) years*
Age range at enrolment		27-84 years
M, F		38-84, 27-75 years
Cancer assessment		Patient total
Solid organ tumour		74
M, F		14, 60
Haematological malignancy		8
M, F		3, 5
Sarcoma		2
M, F		1, 1

M, male; F, female; SD, standard deviation. *p<0.02; Unpaired t-test.

Seventy-four patients were diagnosed with a solid organ tumour of which the most common diagnoses were that of breast, lung and colorectal cancer (see Figure 2.1 for a summary outline of diagnoses). Breast cancer was the most frequently observed malignancy (number of patients, n=45), of which 40 patients were diagnosed with an infiltrating ductal carcinoma and five were diagnosed with an infiltrating lobular carcinoma. A total of 13 lung cancer patients were recruited with most patients presenting with a diagnosis of non-small cell lung cancer (number of patients, n=9). Ten patients with colorectal cancer (adenocarcinoma; number of patients, n=9) and seven patients with lymphoma (Hodgkin's; number of patients, n=2, Non-Hodgkin's; number of patients, n=5) were also recruited to the cohort.

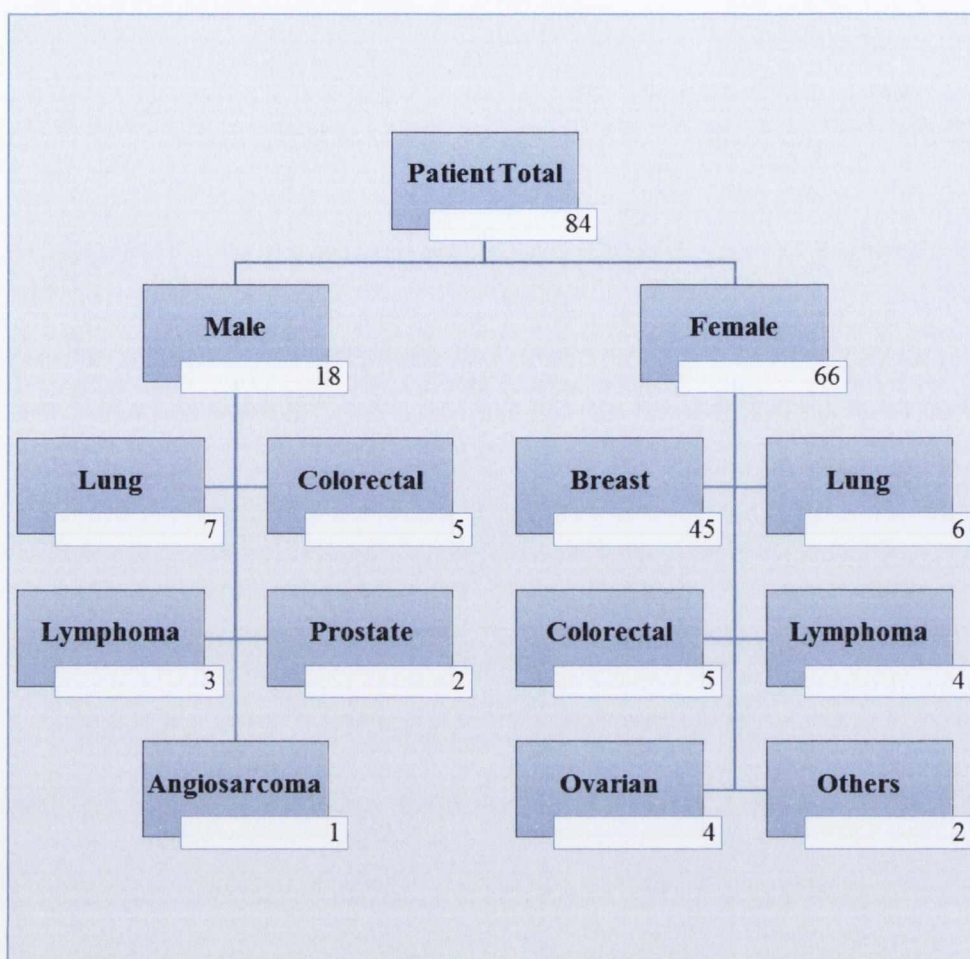


Figure 2.1 Summary outline of study cohort diagnostic characteristics. The study cohort was predominantly female, with breast cancer the most common diagnosis. Others: Leukaemia (number of patients, n=1), Leiomyosarcoma (number of patients, n=1).

2.2.3 Treatment characteristics of the study cohort

A total of 26 different chemotherapeutic regimens were utilised by the study cohort, with breast cancer treatments the most frequently employed (Table 2.3). The most commonly utilised chemotherapeutic agents included cyclophosphamide (number of patients, n=42) and paclitaxel (number of patients, n=38). A total of 38 patients received an anthracycline agent (doxorubicin; number of patients, n=37) as part of their chemotherapeutic regimen. Furthermore, carboplatin was utilised in the chemotherapeutic regimen of 22 patients.

Table 2.3 Treatment regimens utilised among the study cohort.

Treatment regimen	n	Treatment regimen	n	Treatment regimen	n
ACT	14	TC	3	Folfox/Bevacizumab	1
T-FAC	8	Folfox/Cetuximab	3	TH	1
ACTH	7	Carboplatin/Premetrexed	3	THP	1
TCH	5	CMF	3	Carboplatin/Gemcitabine	1
Carboplatin/Paclitaxel	5	Docetaxel	2	5-FU	1
Carboplatin/Navelbine	4	Paclitaxel	2	M-FLOX	1
Carboplatin/Etoposide	4	ABVD	2	Doxorubicin (SA)	1
Folfox	4	TEH	2	DA 3+ 10	1
R-CHOP	4	CHEOP	1		

(n=number of patients). **ABVD**, doxorubicin, bleomycin, vinblastine, dacarbazine; **ACT**, doxorubicin, cyclophosphamide, paclitaxel; **ACTH**, doxorubicin, cyclophosphamide, paclitaxel, trastuzumab; **CHEOP**, cyclophosphamide, doxorubicin, vincristine, etoposide, prednisolone, ranitidine; **CMF**, cyclophosphamide, methotrexate, 5-fluorouracil; **DA 3+10**, daunorubicin, cytarabine; **Folfox**, oxaliplatin, calcium leucovorin, fluorouracil; **5-FU**, 5-Fluorouracil; **M-FLOX**, calcium leucovorin, oxaliplatin, 5-fluorouracil; **R-CHOP**, rituximab, vincristine, doxorubicin, cyclophosphamide, prednisolone; **SA**, single agent; **TC**, docetaxel, cyclophosphamide; **TCH**, trastuzumab, carboplatin, docetaxel; **TEH**, docetaxel, cyclophosphamide, trastuzumab; **T-FAC**, paclitaxel, fluorouracil, cyclophosphamide, doxorubicin; **TH**, paclitaxel, trastuzumab; **THP**, paclitaxel, trastuzumab, pertuzumab.

2.2.4 Clinical characteristics of the study cohort

A mean patient weight of 72 kg (SD; 16.17) was identified prior to commencement of chemotherapy with no significant changes during the course of treatment ($p>0.05$; 1-way analysis of variance (ANOVA)). The mean patient body surface area was 1.76 m² (SD; 0.20) before treatment with no changes observed throughout the course of chemotherapy ($p>0.05$; Repeated-measures ANOVA).

2.3 Statistical analysis of study results

Statistical analysis of the study data was performed using GraphPad Prism (Graph Pad, USA). Results were tested for normality using the D'Agostino-Pearson omnibus normality test. Where data was found to be normally distributed, parametric tests were used for assessment; unpaired t-test, 1-way ANOVA, repeated measures ANOVA and the Pearson product moment correlation coefficient (two-tailed correlation). Results were presented as mean $\pm$ SD. In the case of data which was not normally distributed, non-parametric statistical tests were used in the assessment and included; Wilcoxon signed-rank test (paired t-test), Mann-Whitney U test (unpaired t-test), Spearman's Rank-Order Correlation (two-tailed correlation), Kruskal-Wallis H test (one-way ANOVA) and Friedman test (repeated measures ANOVA). Where necessary, post-hoc analysis was performed using Dunn's multiple comparison test. Results were presented as median $\pm$ IQR (Interquartile range). All results examined were classified as statistically significant when $p<0.05$.

Chapter 3

Development and validation of a novel mass spectrometry method for aldosterone analysis

.....

3.1. Introduction

Cardiac remodelling has been implicated in the pathophysiology of chemotherapy-mediated cardiac injury and is thought to be influenced by many factors including aldosterone (Akpek *et al.*, 2015). However, poor sensitivity and specificity in quantifying this biomarker using current RIA and immunoassay techniques have thus far precluded its evaluation in the detection of cardiac injury. The development of an LC-MS/MS method to quantify aldosterone provides the opportunity for a sensitive and specific means of routinely assessing its role in cardiac remodelling.

The process of LC-MS/MS has emerged as a more sensitive and specific means of quantifying aldosterone when compared with RIA and immunoassay, demonstrating the advantages of using small sample volumes and rapid turnaround times (Keevil, 2013), which promotes its utility in clinical practice. Aldosterone ($C_{21}H_{28}O_5$) is a 21-carbon corticosteroid with a molecular mass of 360.444 Da (LogD (pH: 7.4): 3.85 and LogP: 0.73). It has previously been quantified using a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) assay with quantification in the clinically optimal ranges of 69.4-5,548 pmol/L (Taylor *et al.*, 2009). Various sample preparation techniques including SPE (Hinchliffe *et al.*, 2013), LLE (Ray *et al.*, 2014) and SLE (Owen & Keevil, 2013) have improved sample preparation methods facilitating greater efficiency and specificity in preparation and assessment using LC-MS/MS.

In this study, an LC-MS/MS assay was developed with subsequent assay evaluation and validation using a second mass spectrometer with improved sensitivity, as measured by quantifier and qualifier ion signal intensity. This facilitated the quantification of aldosterone and evaluation of cardiac remodelling, indicative of cardiac injury.

The aims of this study were:

- To evaluate the sample preparation methods of SPE, LLE and SLE in order to achieve optimum levels of sensitivity and specificity to quantify aldosterone using LC-MS/MS
- To optimise liquid chromatography conditions and instrument parameters of a Waters Xevo® TQ mass spectrometer, with the objective of attaining levels of sensitivity at clinically relevant concentrations of aldosterone
- To validate the assay for use in the assessment of serum samples collected in the oncology cohort on a sensitive mass spectrometer (Waters Xevo® TQS mass spectrometer) using an automated sample preparation method

3.2. Methods (Assay procedure 1)

An aldosterone LC-MS/MS assay was initially developed using a Waters Xevo® TQ tandem quadrupole mass spectrometer coupled with a Waters Acquity® ultra performance liquid chromatography (UPLC) system (Waters, Hertfordshire, UK), which was operated in negative ESI mode (ESI-).

3.2.1 Preparation of tuning stocks, internal standard, calibrators and spiked serum pools

A primary stock solution of 1 mg/mL (2.77×10^9 pmol/L) aldosterone (Cerilliant, Sigma-Aldrich, Germany) in methanol (Fischer Scientific, Ireland) was used to prepare a 200 ng/mL working stock solution for mass spectrometer tuning (Figure 3.1).

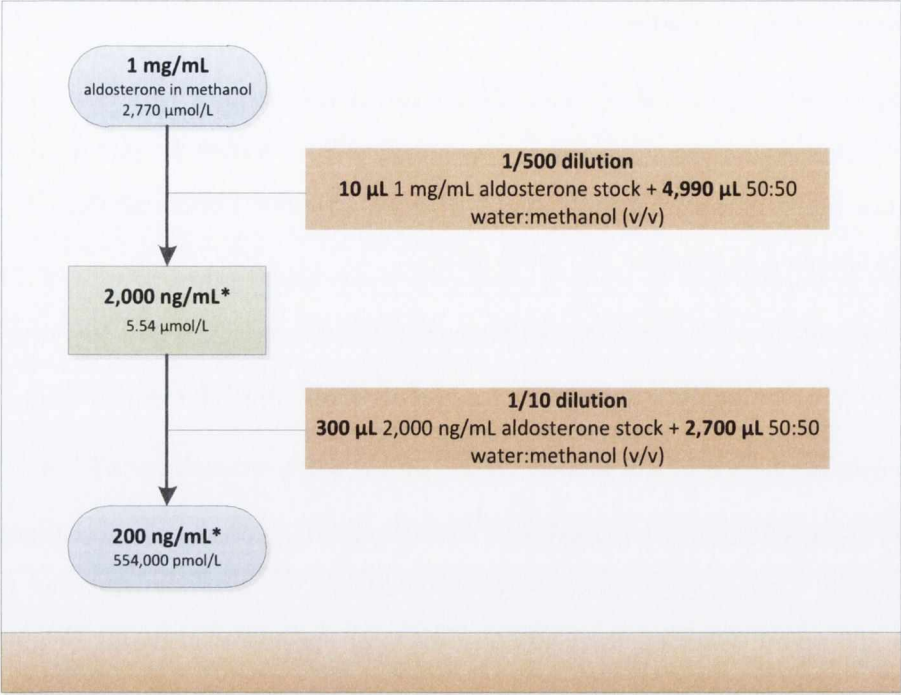


Figure 3.1 Dilution protocol for aldosterone (554 nmol/L) tuning solution.

*Aldosterone in 50:50 water:methanol (v/v).

A working stock of 0.2 ng/mL (554 pmol/L) aldosterone in 70:30 water:methanol (v/v) was prepared for use in tuning optimisation experiments (Figure 3.2).

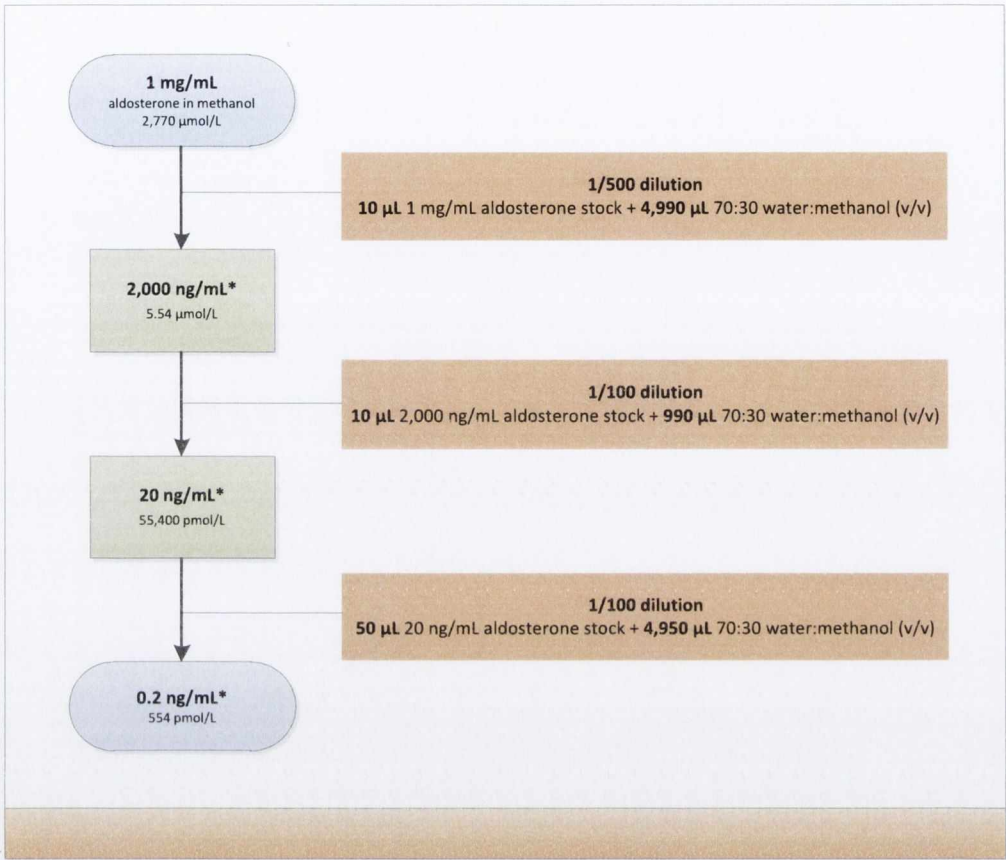


Figure 3.2. Dilution protocol for aldosterone (554 pmol/L) tuning optimisation solution. *Aldosterone in 70:30 water:methanol (v/v).

An LC-MS/MS system suitability test solution, consisting of a 0.1 ng/mL (277 pmol/L) aldosterone in 60:40 water:methanol (v/v) was prepared for assay performance assessment (Figure 3.3).

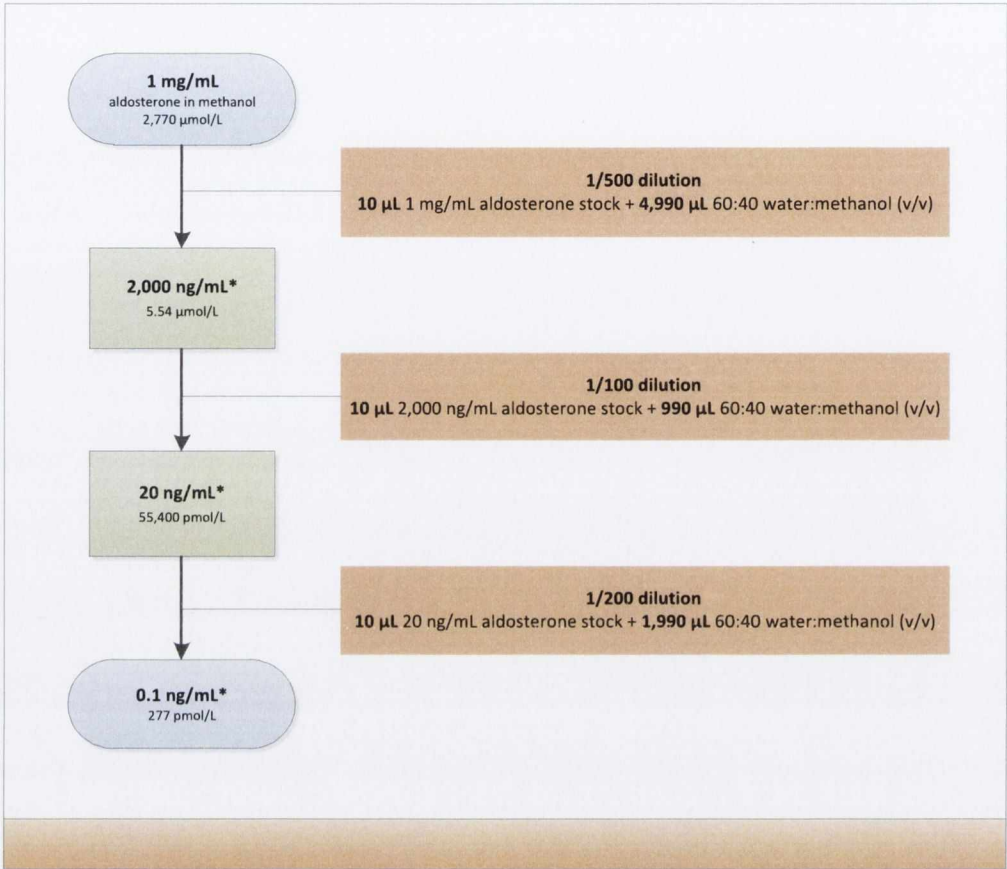


Figure 3.3 Dilution protocol for LC-MS/MS system suitability test solution.

*Aldosterone in 60:40 water:methanol (v/v).

The internal standard, aldosterone- $[\text{}^2\text{H}_7]$ (IsoSciences, USA), was solubilised in methanol and prepared to a final working stock concentration of 1 ng/mL in 50:50 water:methanol (v/v) (Figure 3.4).

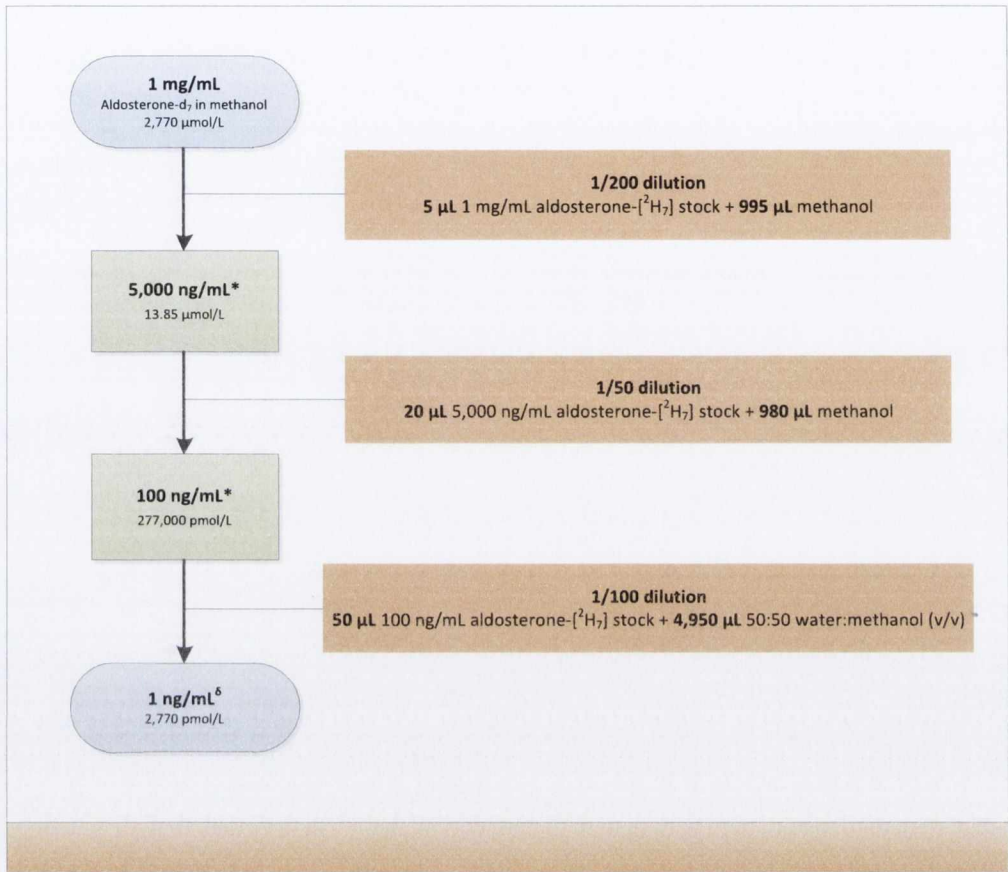


Figure 3.4 Preparation of aldosterone- $[\text{}^2\text{H}_7]$ internal standard. *Aldosterone in methanol; $^\delta$ Aldosterone in 50:50 water:methanol (v/v).

Calibrators were prepared in phosphate buffered saline with 0.1% bovine serum albumin to concentrations of 55, 111, 222, 554, 1,385 and 2,770 pmol/L aldosterone. Spiked serum pools were prepared using redundant serum samples, centrifuged at 3,500 g for 10 minutes at room temperature (Eppendorf 5810 centrifuge; Eppendorf, UK) and pooled in 20 mL polypropylene vials (Sarstedt, Germany). Serum pools were spiked using the calculations from the protocols in Figure 2.2 or Figure 2.3 depending on the required concentration.

3.2.2 Assessment of sample preparation methods

Numerous sample preparation methods including SPE (Hinchliffe *et al.*, 2013), LLE (Ray *et al.*, 2014) and SLE (Owen & Keevil, 2013) have previously been demonstrated as effective techniques to remove lipids and proteins present in patient samples prior to aldosterone assessment using LC-MS/MS. The objective of investigating each sample preparation technique was to achieve sufficient levels of sensitivity by obtaining the greatest signal to noise (S/N) values for quantifier and qualifier ions.

An LLE extraction was evaluated using replicates of pooled serum, spiked to a concentration of 554 pmol/L using MTBE (Thermo Fisher, UK) as the extraction solvent (Figure 3.5).

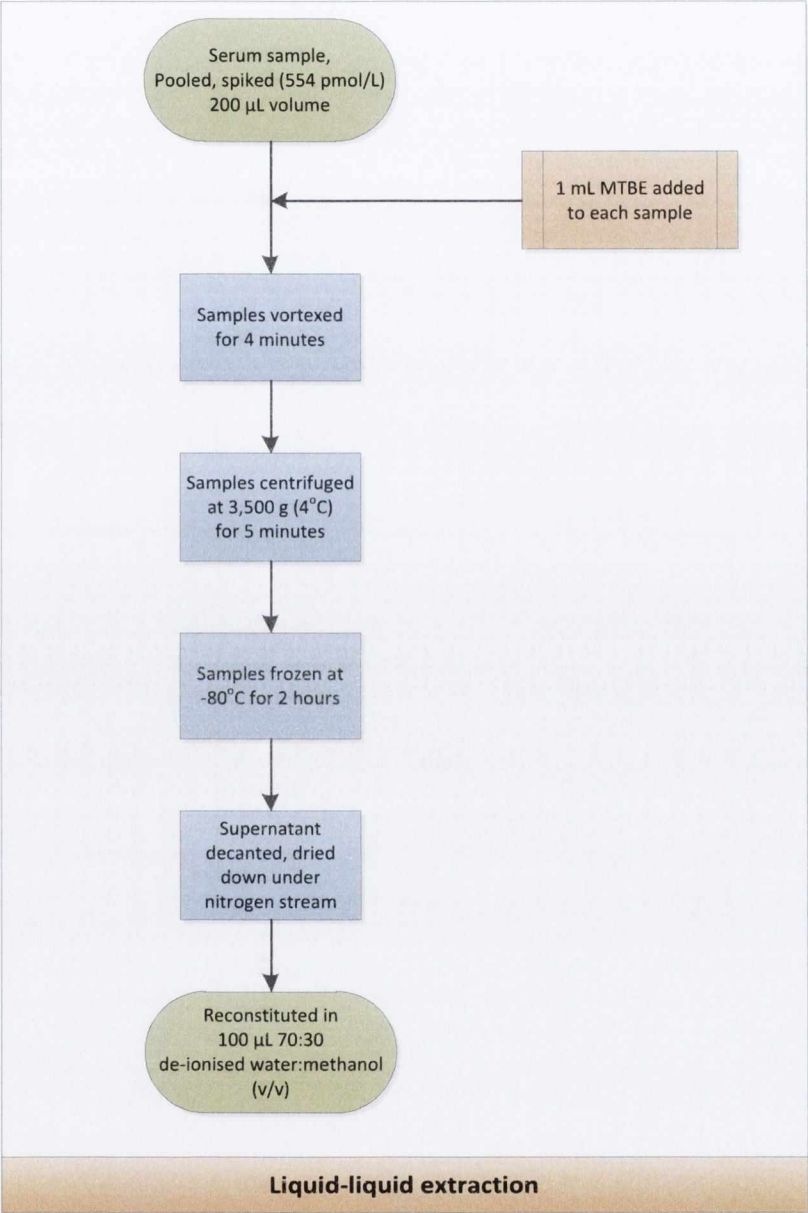


Figure 3.5 LLE sample preparation protocol for aldosterone analysis. MTBE, methyl tert-butyl ether.

A supported liquid extraction procedure using pooled, redundant patient serum spiked with varying concentrations of aldosterone (111, 139, 277, 554 and 1,108 pmol/L) was performed using Biotage SLE+ plates (Biotage, Hertfordshire, UK) (Figure 3.6). DCM (Sigma Aldrich, UK) and MTBE were compared as elution solvents.

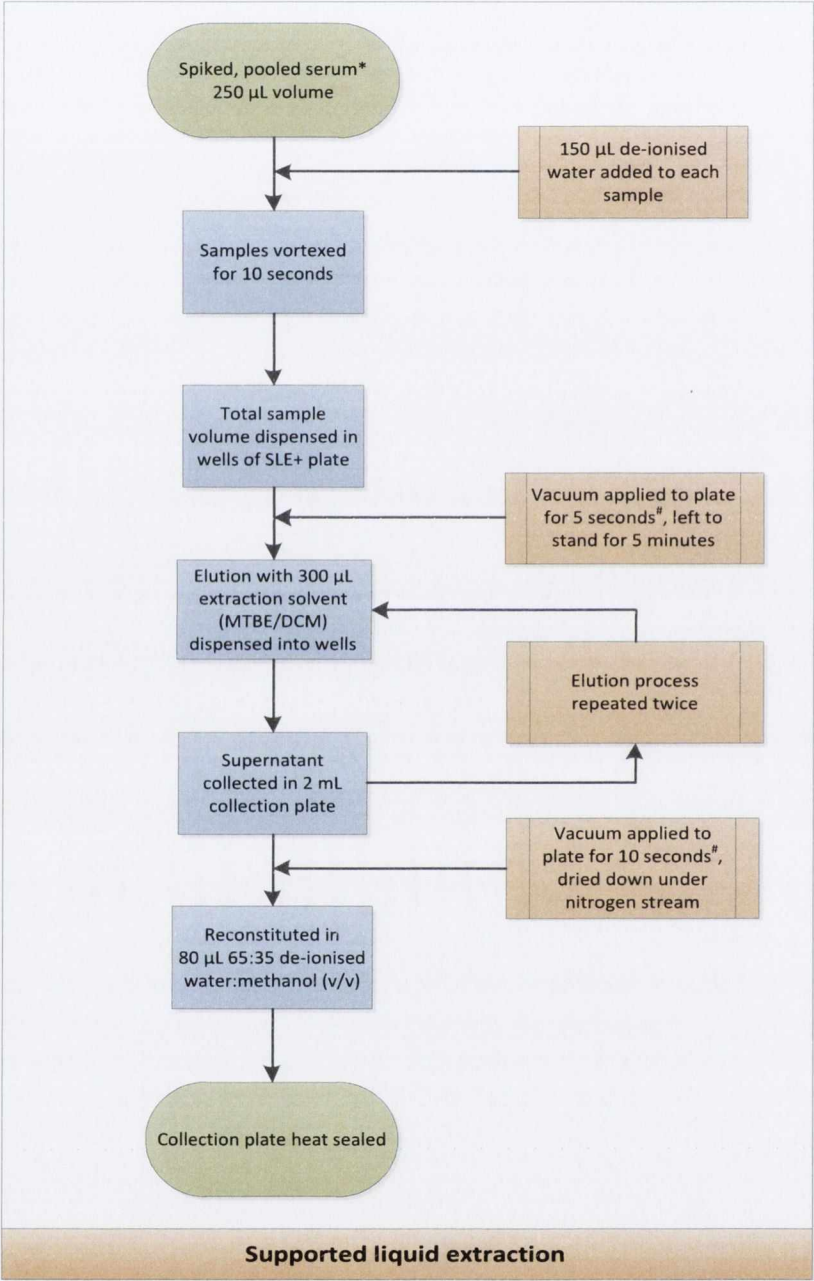


Figure 3.6 SLE sample preparation protocol for aldosterone analysis. DCM, dichloromethane; MTBE, methyl tert-butyl ether. # Medium speed vacuum setting.

A solid phase extraction protocol was examined using a Waters Oasis® mixed mode anion exchange (MAX) µelution plate in the analysis of prepared calibrators and QC (see section 3.2.1) (Figure 3.7). A Tecan Freedom Evo 100 (Tecan, Switzerland) was used to perform sample pre-treatment and elution.

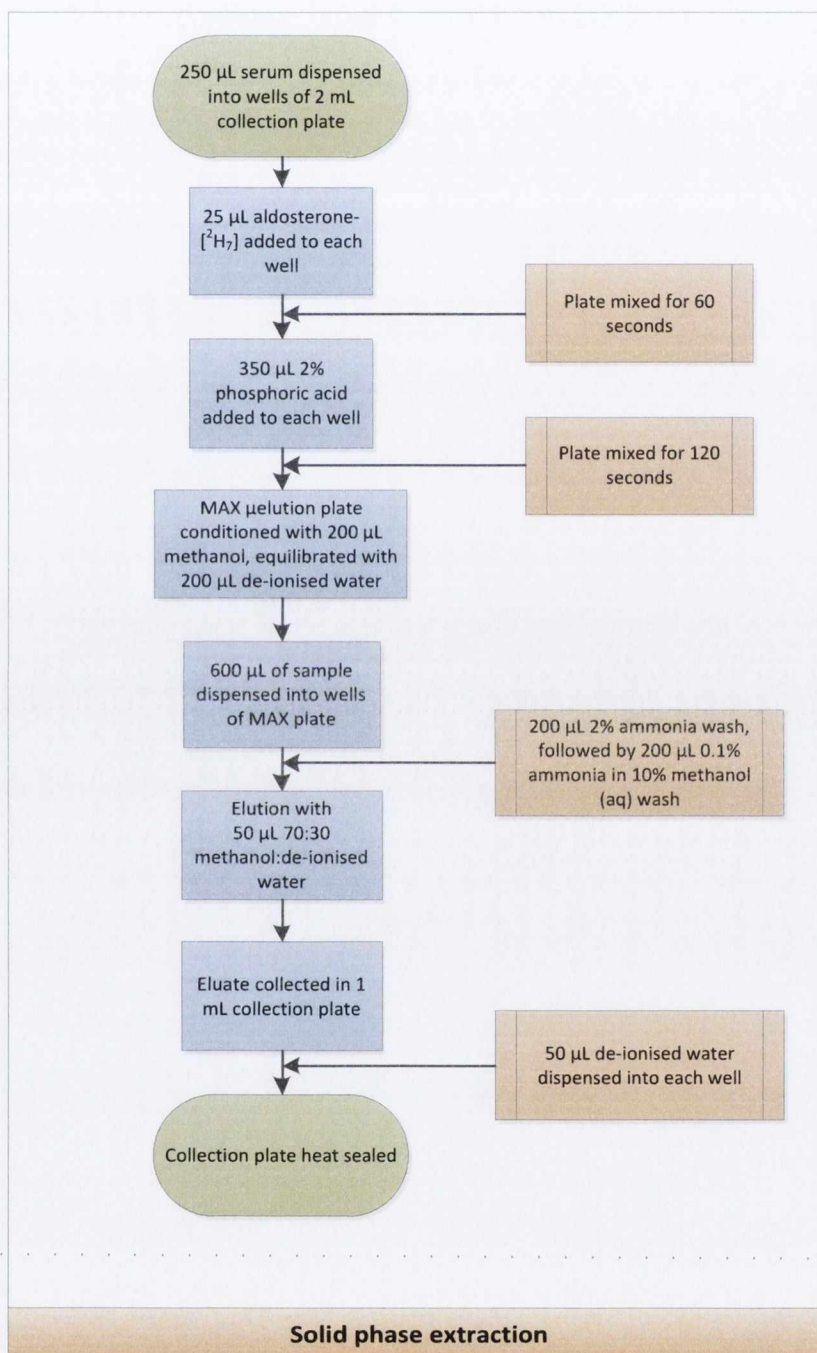


Figure 3.7 SPE sample preparation protocol for aldosterone analysis. MAX, mixed-mode anion exchange.

3.2.3 Optimisation of liquid chromatography settings

This assay was developed using a Waters Acquity® UPLC system. A KrudKatcher UPLC In-Line filter 0.5 μm x 0.1016 mm (Phenomenex, UK) was coupled to the columns evaluated. A Phenomenex Kinetex PFP (pentafluorophenyl; functional group in column), 100 mm x 2.1 mm, 2.6 μm column (Phenomenex, UK) and a CSH C18, 50 mm x 2.1 mm, 1.7 μm column (Waters, UK) were assessed using injections of aldosterone (554 pmol/L in 70:30 de-ionised water: methanol (v/v)) evaluated under variable mobile phase conditions.

The composition of mobile phases A and B, used also as a weak and strong needle wash respectively (weak: strong, 300:100 μL) was varied and examined in order to achieve improved chromatography and enhance sensitivity. Mass spectrometry grade de-ionised water (18 Ω , mobile phase A) (Millipore, UK) and methanol (mobile phase B) (Fischer Scientific, UK) were initially evaluated as suitable preparations. The addition of various concentrations of formic acid (Thermo Fisher, UK) 0.1%, 1% in de-ionised water; mobile phase A and 0.1%, 1% in methanol; mobile phase B), acetic acid (Sigma Aldrich, UK) (1 mM, 10 mM in de-ionised water; mobile phase A and 1 mM, 10 mM in methanol; mobile phase B) and ammonium formate (Sigma-Aldrich, UK) (2 mM in de-ionised water; mobile phase A and methanol; mobile phase B) were prepared and assessed.

The needle injection volume, which designates the volume of sample injected through the column, was investigated in partial and full loop injection modes. A partial loop injection, characterised by an injection volume of 30 μL was compared with a full loop injection of 50 μL . Injection to injection carry-over was investigated through assessment of repeated injections of 5540 pmol/L aldosterone prepared in 70:30 water:methanol (v/v). A 300:100 μL weak: strong needle wash was performed over 30, 60, 90 and 120 second runs following injection of the 5,540 pmol/L sample and followed by solvent blank injections. Experiments were replicated using a 600:200 μL weak: strong needle wash and compared with smaller needle wash volumes.

Following sample injection, chromatographic separation was performed using a gradient elution (Table 3.1). The flow rate was set to 0.45 ml/min with a column temperature of 45°C for the duration of the elution, comprising a sample injection run time of 4.7 minutes.

Table 3.1 Chromatography conditions for each sample injection.*

Time (min)	Mobile phase A Mass spectrometry grade water (%)	Mobile phase B Methanol (%)	Gradient curve
Initial	70	30	Initial
0.2	70	30	1
3.2	35	65	6
3.7	5	95	11
4.7	70	30	11

* Sample injection volume set to 37.5 μ L; Partial loop with needle overfill.

3.2.4 Tuning and optimisation of MS/MS parameters

Mass spectrometry tuning was performed using a 554 nmol/L tuning solution of aldosterone, (see Figure 3.1) to identify the precursor and product ions. Multiple reaction monitoring mode (MRM) was selected for aldosterone precursor ion assessment, with capillary and cone voltage, ion energy and low and high resolution settings (LM 1 and HM 1) adjusted to achieve the optimum unit mass resolution (UMR) of 0.75 Da at full width-half maximum. The mass value at apex of the peak was recorded for the parent ion. To identify quantifier and qualifier product ions for aldosterone in MRM mode, the collision gas (argon) was switched on. The collision energy and ion energy (MS 2) were adjusted, along with the parameters of the low and high resolution settings (LM 2 and HM 2) to achieve the optimum UMR of 0.75 Da for qualifier ions. A scan of the product ion masses was performed to assess the accuracy of the tuning procedure.

Aldosterone was detected in MRM mode. The MRM quantifier and qualifier ion transitions for aldosterone had a mass-to-charge ratio (m/z) of 359.2>189 and m/z 359.2>331.15 respectively with a retention time of 2.82 minutes.

The mass spectrometer parameters were optimised using a 554 pmol/L aldosterone tuning solution (see Figure 3.2) to improve detection sensitivity. The source temperature was set at 150°C and cone gas flow was set to 150 L/h. Collision gas flow was set to 0.15 ml/min. Cone voltage and collision energy, capillary voltage and desolvation gas flow were optimised independently before a combined optimisation of parameters was performed (Table 3.2). Each parameter value to be further investigated in this combination experiment was paired with each selected setting from the other instrument parameters to achieve optimum instrument settings.

Table 3.2 Instrument values for initial parameter optimisation.

Cone voltage (V)	Collision energy (eV)	Capillary voltage (kV)	Desolvation gas flow (L/Hr)	Desolvation temperature (°C)
25*	15*	1.0	975	500
28*	18*	1.2*	1000*	525
30*	21	1.4	1025*	550
32		1.6*		

* Parameter values evaluated in combination with other parameter variables

3.3 Materials and Methods (Assay procedure 2)

Owing to the sub-optimal sensitivity attained using assay procedure 1, a second assay procedure was investigated which had been developed utilising a tandem quadrupole mass spectrometer with greater sensitivity (Waters® TQS tandem quadrupole mass spectrometer), achieved by using a larger cone orifice facilitating the passage of greater numbers of ions into the mass spectrometer and also by using StepWave ion optic technology which assists in the removal of unwanted neutral molecules and noise. This method also employed an efficient, semi-automated sample preparation protocol which suitably prepared samples for aldosterone assessment.

This method was performed using a Waters Acquity® UPLC I-class system (Waters, Manchester) coupled with the Waters Xevo® TQS mass spectrometer operating in negative ESI mode.

3.3.1 Preparation of calibrators, quality controls, internal standard and samples

Calibrators were prepared in stripped human serum (Golden West Biologicals, USA) to concentrations of aldosterone (Cerilliant, USA) ranging from 42 to 4,161 pmol/L. Quality controls (QC) were prepared in-house using pooled plasma (SeraLab, UK) to final concentrations of 99, 500 and 2,000 pmol/L. The internal standard used was aldosterone- $^{2}\text{H}_4$ (IsoSciences, PA). Additional external quality control samples (UKNEQAS) were also used to evaluate assay accuracy.

Oncology samples which were frozen prior to analysis were thawed, homogenously mixed and centrifuged at 3,500 g for five minutes before pre-treatment by SPE and subsequent analysis using the Waters Xevo® TQS tandem quadrupole mass spectrometer. This preparation protocol was adopted for assessment of all samples in the study.

3.3.2 Preparation of samples using SPE

A semi-automated SPE protocol using a Tecan Freedom Evo 100/4 liquid handler (Tecan, Switzerland) was performed using a Waters Oasis MAX μ elution plate to facilitate sample pre-treatment and elution (Figure 3.8).

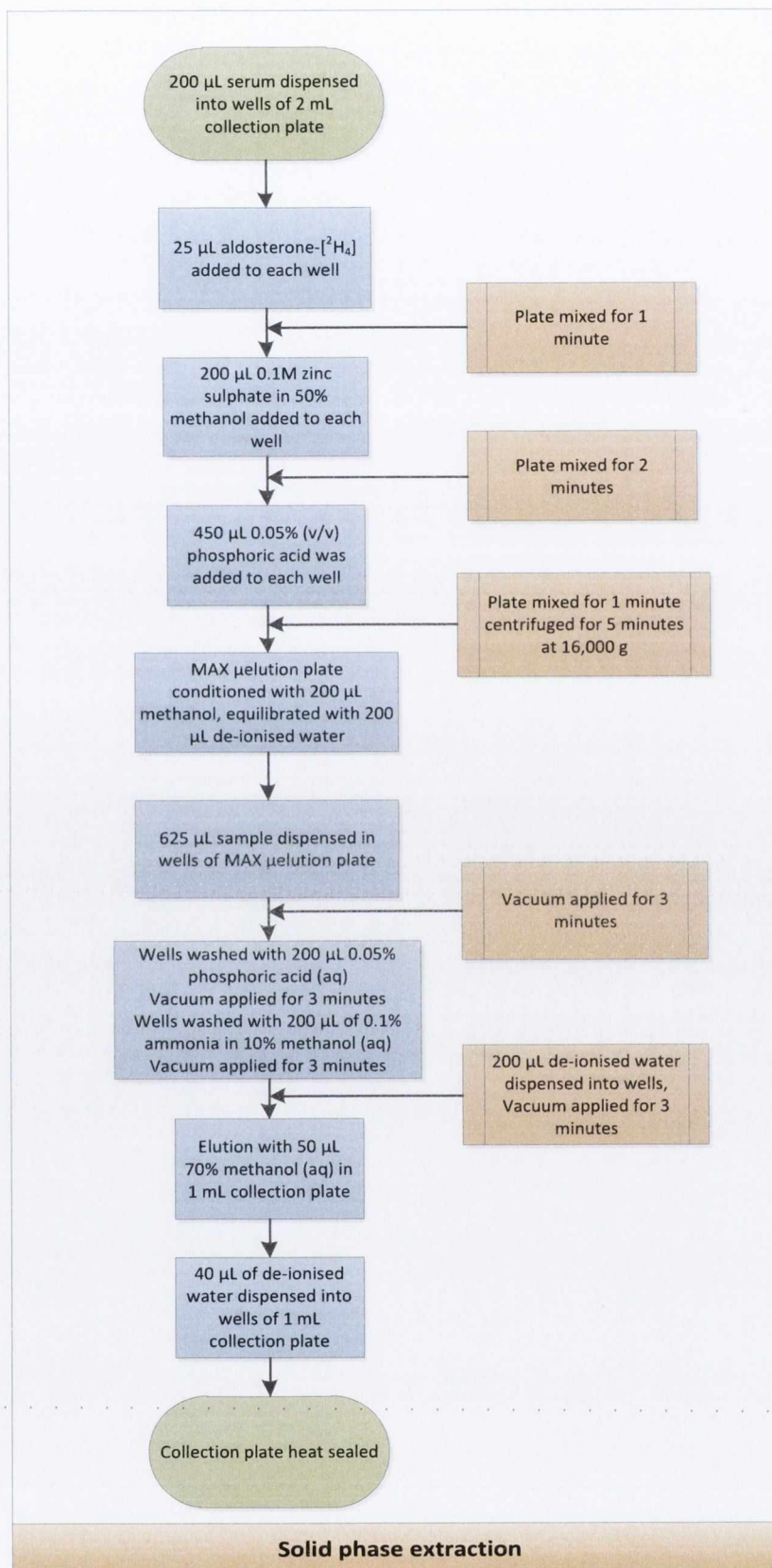


Figure 3.8 SPE sample preparation protocol for aldosterone analysis.

3.3.3 Assessment of sample suitability and minimum volume requirements

Serum (collected in gel separator tubes) and plasma sample matrices were evaluated for the level of concordance in quantifying aldosterone and assessed for the potential interferences by components of serum gel tubes. A 500 μ L aliquot of a serum and plasma sample taken on the same day from a total of 40 patients was collected, centrifuged at 3,500 g for five minutes and stored at -20°C until analysis. Samples were thawed, homogenously mixed and centrifuged at 3,500 g for five minutes before pre-treatment by SPE and subsequent analysis using the Xevo® TQS tandem quadrupole mass spectrometer.

The minimum sample volume required for assessment was evaluated using a medium level quality control sample (480 pmol/L) at four different volumes (200, 220, 250 and 300 μ L). Samples were prepared in triplicate, thawed, homogenously mixed and centrifuged at 3,500 g for five minutes prior to sample preparation and interchangeably measured during the course of the analytical run.

3.3.4 Liquid chromatography evaluation

Samples were injected onto a Waters Acquity® in-line filter and frit unit coupled with a Cortecs UPLC® C₁₈ 100 mm x 2.1 mm, 1.6 μ m column (Waters, Manchester, UK), maintained at 45°C on a Waters Acquity® UPLC I-Class system. The flow rate was set at 0.4 ml/min during each 4-minute chromatographic run (Table 3.3).

Table 3.3 Chromatographic conditions for each sample injection.*

Time (min)	Mobile phase A	Mobile phase B	Gradient curve
	Mass spectrometry grade water (%)	Methanol (%)	
Initial	60	40	Initial
1	60	40	6
2	40	60	6
2.3	5	95	11
2.8	60	40	11

* Sample injection volume set to 25 µL; Partial loop with needle overfill.

3.3.5 Tandem mass spectrometry assessment

Samples were assayed using a Waters Xevo® TQS operating in negative ESI mode. Capillary and cone voltage were set at 2.4 kV and 55 V respectively with a collision energy of 18eV. Cone gas flow was set to 150 L/h. Collision gas flow was set to 0.15 ml/min while desolvation gas flow was set to 1,000 L/h. Desolvation temperature was set to 600°C, with a source temperature of 150°C. Using a dwell time of 0.1 seconds per channel, the precursor ion was identified with a mass to charge ratio (m/z) of 359.2 and the quantifier and qualifier product ions were identified with an m/z of 189.2 and 297.2 respectively. The qualifier quantifier ion ratio was determined to be 2.70 ± 0.48 . The internal standard aldosterone-[$^4\text{H}_2$] had an m/z of 190.2.

3.3.6 Validation of the LC-MS/MS assay

The validation of the assay was performed based on guidelines published by Honour (Honour, 2011). The limit of quantification (LOQ) was determined by repeated assessment of calibration standards prepared in stripped human serum to varying concentrations where the lowest value to achieve an S/N ratio greater than 10:1 on 10 occasions. The LOD was calculated as the smallest detectable peak distinguishable from baseline noise (with an S/N ratio greater than 3:1). Assay linearity was determined by analysis of six calibration curves which comprised calibrators within the concentration range of 42 to 4,161 pmol/L. Weighted linear regression was utilised to confirm linearity with an r^2 (correlation coefficient) value of greater than 0.99 deemed satisfactory for analysis.

Assay precision was evaluated during analysis of oncology samples by measurement of three QC samples assessed in duplicate over a 5-day period. The accuracy of the assay was calculated following duplicate measurement of 35 external QC (NEQAS, National External Quality Assessment Scheme) samples over a 5-day period. Bland-Altman plots were used to compare results with all method mean values. Method comparison with another mass spectrometry assay was performed. The recovery of the assay was determined by assessment of

pooled, plasma QC samples which were subsequently spiked with concentrations of 100, 500 and 2,000 pmol/L aldosterone and re-analysed.

The specificity of the assay was determined by evaluation of the response following addition of each of the steroids individually to a common pooled serum sample which had a previously determined aldosterone concentration. The nine steroids assessed were corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol, 21-deoxycortisol, 18-hydroxycorticosterone, prednisone and prednisolone. Matrix effects were investigated by assessment of plasma samples spiked with aldosterone post-extraction evaluated against samples spiked with equivalent concentrations pre-extraction.

3.3.7 Assessment of external QC during sample analysis

UKNEQAS samples (number of samples, n=6) were assessed for comparison with other mass spectrometry-based aldosterone assays during the analysis of samples collected from oncology patients for this study. The standard deviation from the mean derived values for all mass spectrometry users (number of users, n=8) was determined for each sample.

3.4 Results (Assay procedure 1)

3.4.1 Assessment of sample preparation procedures

The assessment of LLE, SLE and SPE methods revealed significant issues in preparing samples for assessment. The evaluation of LLE using pooled serum samples spiked with 554 pmol/L aldosterone revealed sub-optimal levels of sensitivity as seen from the low levels of sensitivity, for the quantification of aldosterone quantifier ion 189, with signal intensity ranging from 1.89×10^3 to 3.67×10^3 , peak-to-peak S/N which ranged from 28 to 47 and with a retention time of 2.87 minutes (Figure 3.9). Such levels of signal intensity would subsequently result in poor assay imprecision, particularly at aldosterone concentrations less than 555 pmol/L. The signal intensity for the qualifier ion 331.15 ranged from 2.74×10^3 to 3.75×10^3 with a peak-to-peak S/N which ranged from 31 to 65. There was peak splitting observed in the chromatography with a maximum of 11 points per peak achieved. In an attempt to eliminate the effect of peak splitting, the dwell time was optimised to 0.080 seconds to try and achieve the optimum of 15 points per peak.

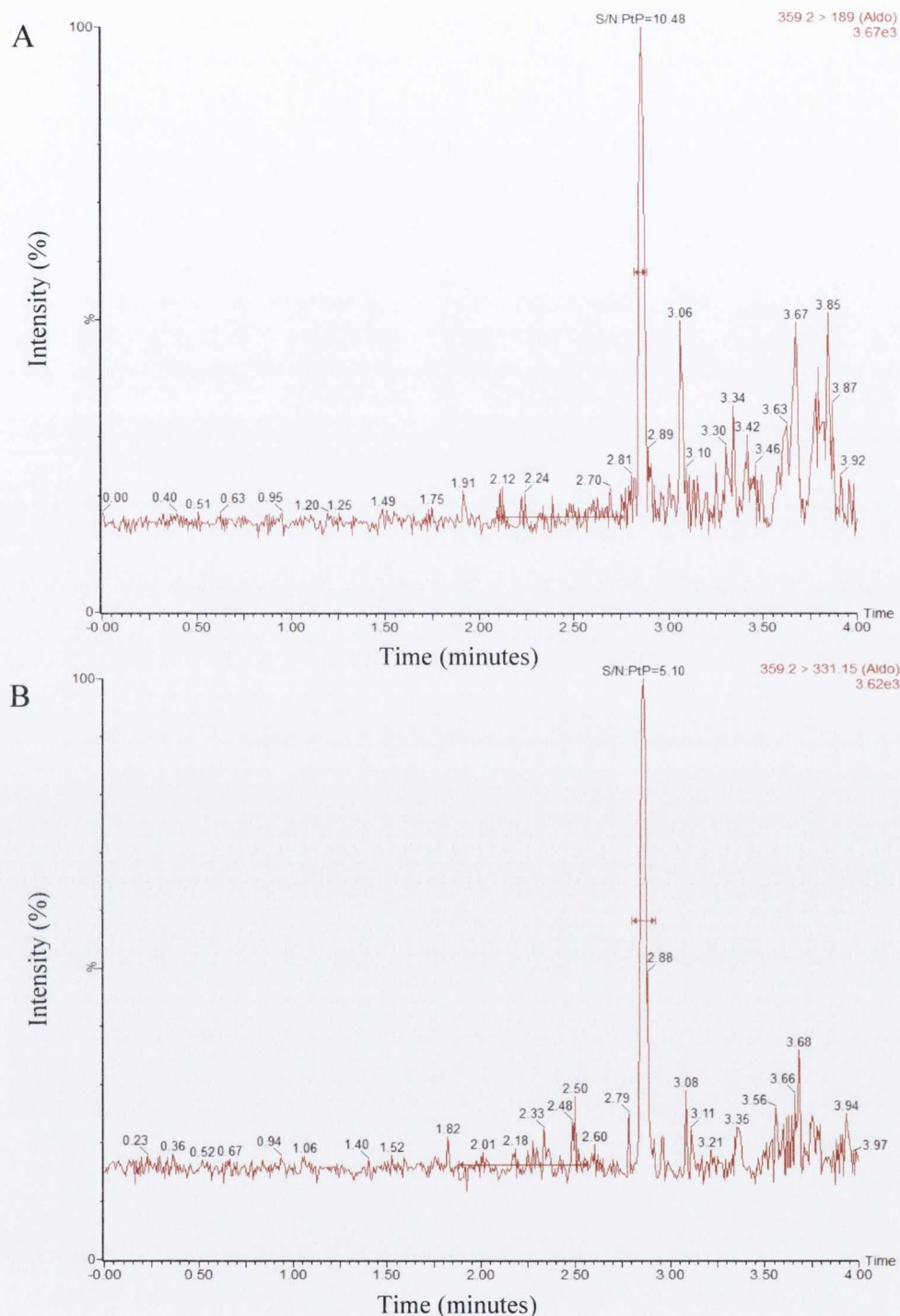


Figure 3.9 Comparison of quantifier (A) (m/z 359.2>189) and qualifier (B) (m/z 359.2>331.15) ion transitions for a spiked (554 pmol/L), pooled, redundant patient serum sample prepared using LLE. Sub-optimal signal intensities were obtained for both ions. Retention time; 2.87 minutes. S/N values were calculated using peak-to-peak assessment as highlighted above with the red arrows.

The SLE sample preparation procedure evaluated using pooled, redundant patient serum samples spiked with varying concentrations of aldosterone demonstrated a faster preparation time when compared with LLE. Use of DCM extraction solvent generated significantly greater signal intensities and S/N values for the quantifier and qualifier ions. DCM extraction of pooled serum samples with 554 pmol/L aldosterone demonstrated a signal intensity for the quantifier ion 189 which ranged from 1.43×10^4 to 1.45×10^4 , a peak-to-peak S/N which ranged from 63 to 77 and a retention time of 2.87 minutes. Serum samples with a concentration of 111 pmol/L aldosterone demonstrated a signal intensity within the range of 6.26×10^3 and 6.56×10^3 , with a peak-to-peak S/N of between 12 and 37.

MTBE extraction also using pooled serum samples spiked with 554 pmol/L aldosterone demonstrated signal intensity values for quantifier ion 189 which ranged from 3.49×10^3 to 4.02×10^3 , a peak-to-peak S/N which ranged from 22 to 23 and a retention time of 2.86, but with multiple smaller peaks identified between 2.85 and 2.89. In a direct comparison with samples extracted using DCM (Figure 3.10), samples spiked with 111 pmol/L aldosterone and extracted using MTBE demonstrated a signal intensity which ranged from 976 to 3.70×10^3 . The peak-to-peak S/N values ranged between 4 and 24 with a retention time of 2.86.

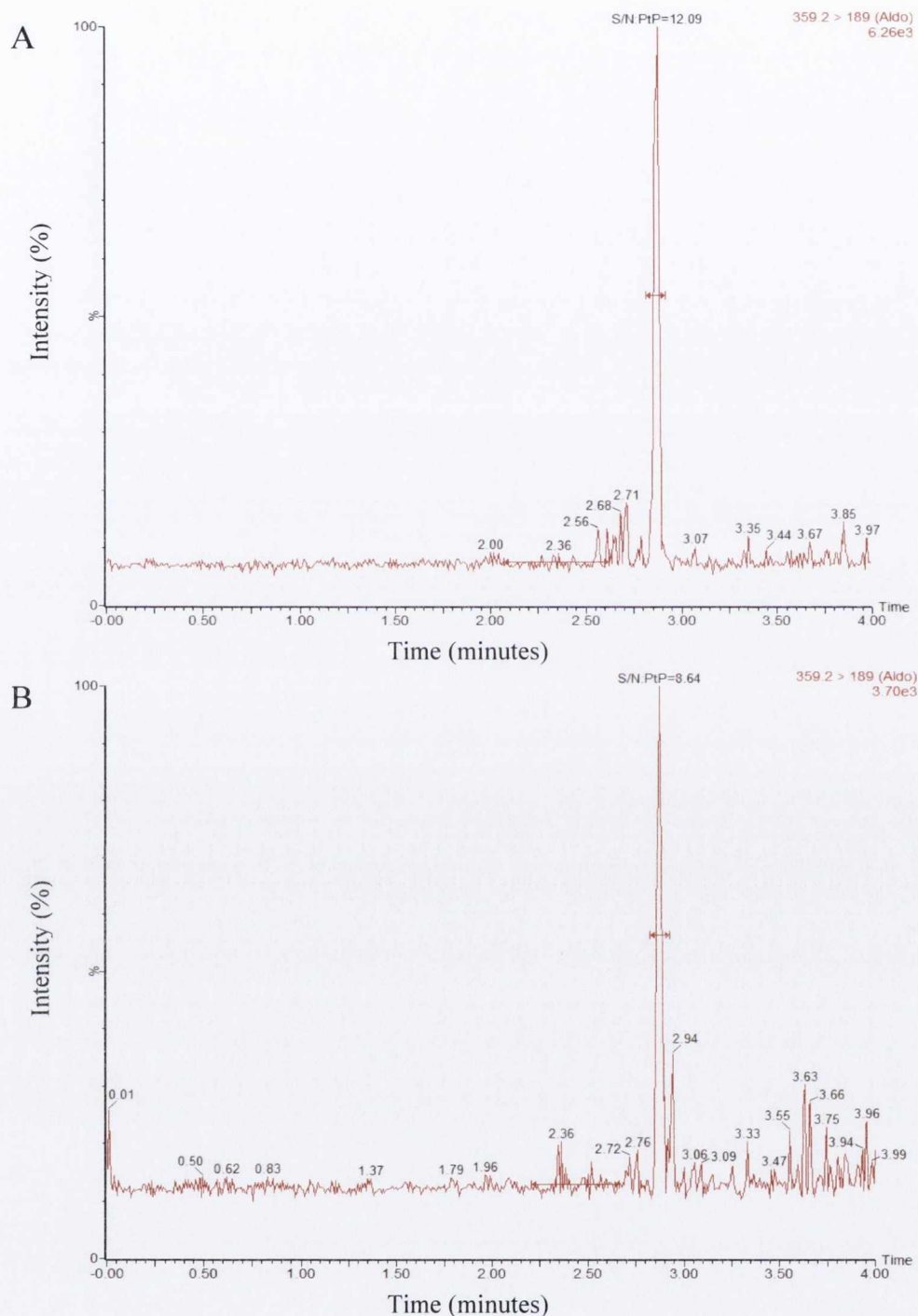


Figure 3.10 Comparison of quantifier (m/z 359.2>189) ion transitions for a spiked (111 pmol/L) pooled, redundant patient serum samples extracted using DCM (A) and MTBE (B) in an SLE procedure. There was a significant reduction in signal intensity and S/N using MTBE as the extraction solvent. Retention time; 2.87 minutes. S/N values were calculated using peak-to-peak assessment as highlighted above with the red arrows.

In an assessment of the SPE procedure developed for the quantification of aldosterone in assay procedure 1 (Figure 3.8), the wells of the Waters Oasis® MAX μ elution plate were obstructed following loading, which prevented completion of the wash and elution steps. This may have been a consequence of the absence of a protein precipitation step in the procedure prior to loading the samples onto the SPE plate. In an evaluation of calibrators prepared in phosphate buffered saline and 0.1% bovine serum albumin, the S/N values obtained for the calibrator of 111 pmol/L were found to be lower than the absolute minimum recommended S/N limit of 10:1, with S/N values of 12 detected using calibrators of 1,385 pmol/L (Figure 3.11). These S/N values obtained within the clinically relevant concentration range for aldosterone were below the minimum required level for assessment and hence prevented the quantification of aldosterone in samples prepared using this SPE method.

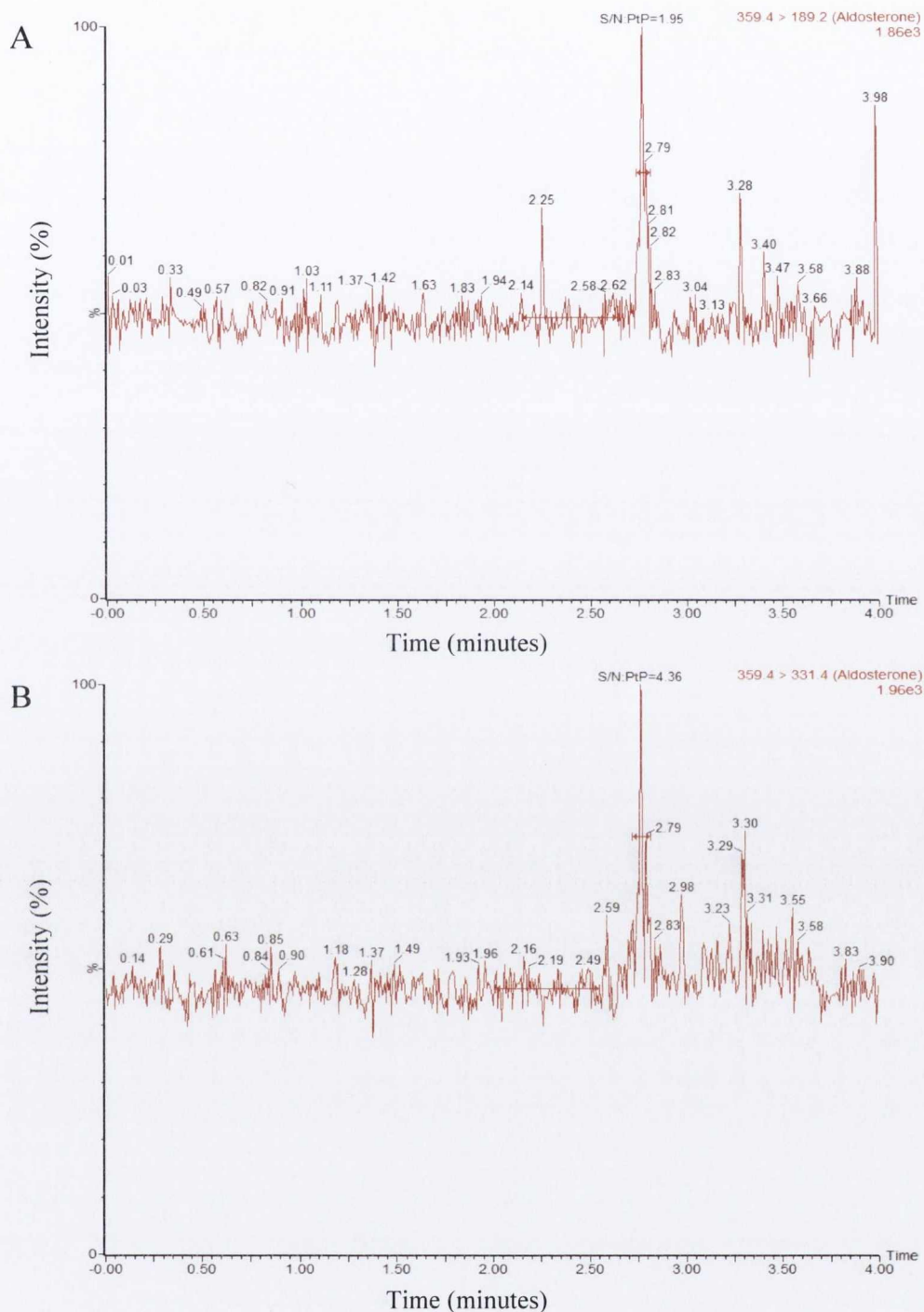


Figure 3.11 Assessment of quantifier (A) (m/z 359.2>189) and qualifier (B) (m/z 359.2>331.15) ions from a 111 pmol/L calibrator prepared in phosphate buffered saline and 0.1% bovine serum albumin extracted using SPE. Retention time; 2.77. S/N values were calculated using peak-to-peak assessment as highlighted above with the red arrows.

3.4.2 Evaluation of liquid chromatography optimisation

The Phenomenex PFP column achieved the greatest sensitivity for quantifying aldosterone, in combination with de-ionised water and methanol as mobile phases A and B respectively. The signal intensity values achieved for the quantifier ion (m/z 359.2>189) were determined between 8.12×10^3 and 1.03×10^4 in an assessment of a 554 pmol/L sample prepared in 30% methanol with a retention time of 2.88 minutes (S/N range; 37 to 50). When compared with the CSH C_{18} column, the maximum signal intensity obtained for the quantifier ion (m/z 359.2>189) using 554 pmol/L aldosterone in 30% methanol (v/v) was 3.54×10^3 with an S/N of 86. In an assessment of mobile phase additives evaluated (formic acid, acetic acid and ammonium formate; see section 3.2.3) for the enhancement of signal intensity and S/N, the maximum quantifier ion (m/z 359.2>189) and qualifier ion (m/z 359.2>331.15) intensity values attained were significantly lower, although S/N values were above the acceptable limits for quantification.

3.4.3 Assessment of mass spectrometer sensitivity

In an evaluation of instrument parameters operating in ESI negative mode, cone voltage was optimised to 28 V and capillary voltage set to 1.6 kV. Collision energy was optimised to 18 eV. Desolvation gas flow set to 1,000 L/h and desolvation temperature was set to 550°C. The low mass resolution setting for MS1 and MS2 was 2.8, with a high mass resolution setting of 15. A dwell time of 0.08 seconds per channel was selected for assessment based on the number of points measured per peak.

Aldosterone at concentrations across the clinically relevant range spiked in a 70:30 water:methanol (v/v) preparation and directly injected onto the LC column facilitated the most efficient means of assessing the sensitivity of the mass spectrometer. In this study, aldosterone prepared at concentrations near the desired lower limit of quantification of 100 pmol/L (139 pmol/L in this assessment) directly injected onto the PFP column did not achieve adequate signal intensity for the quantifier and qualifier ions (Figure 3.12) following optimisation of the mass spectrometer parameters. The S/N values of >10:1 required in a clinical setting to quantify aldosterone were not achieved. As a result, a second

assay procedure was developed to facilitate quantification of aldosterone using a more sensitive instrument and sample preparation procedure.

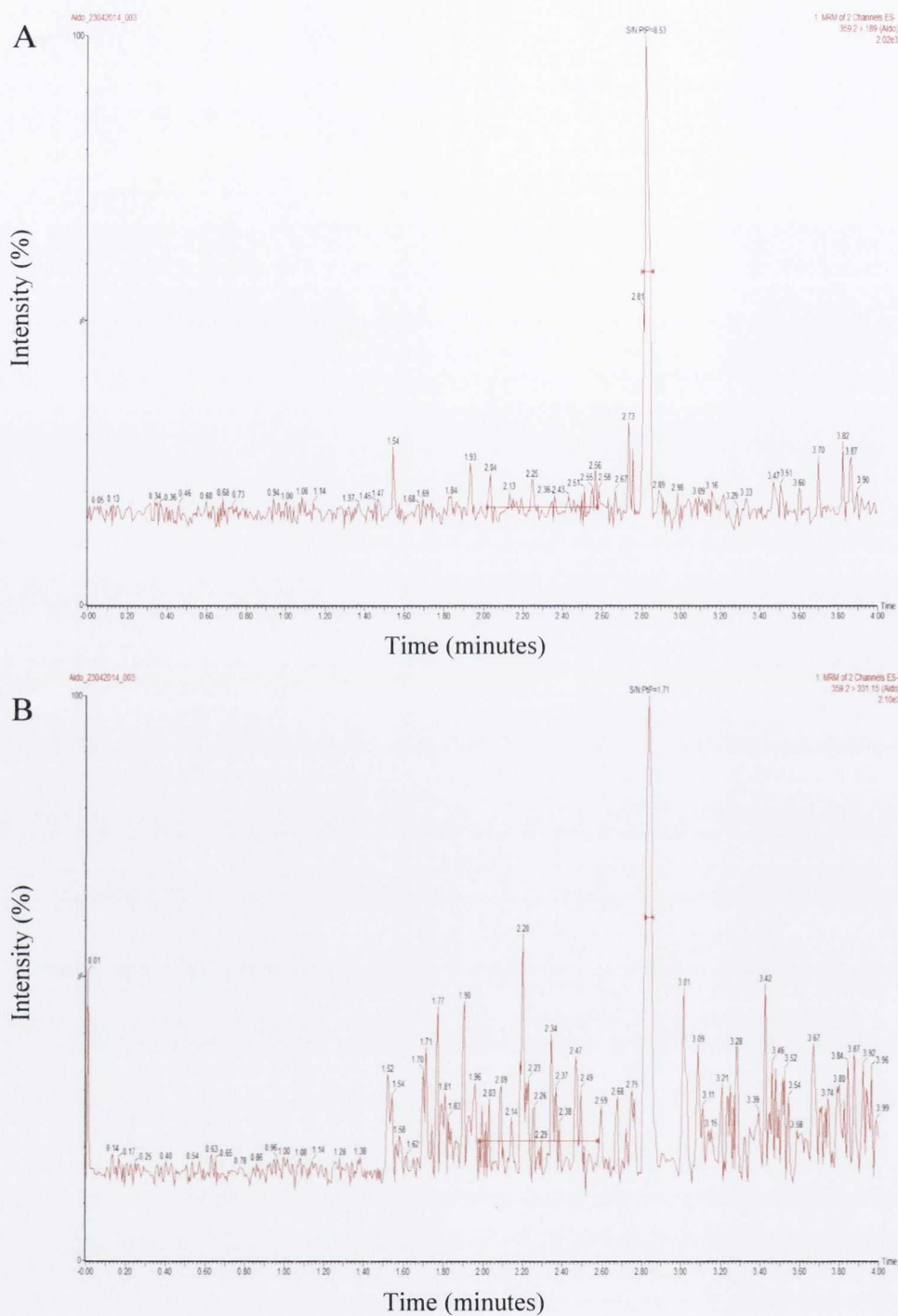


Figure 3.12 Assay procedure 1 quantifier (A) (m/z 359.2>189) and qualifier (B) (m/z 359.2>331.15) ion transitions for 138.5 pmol/L aldosterone prepared in aqueous 30% methanol (v/v). Retention time; 2.87 minutes. S/N values were calculated using peak-to-peak assessment as highlighted above with the red arrows.

3.5 Results (Assay procedure 2)

3.5.1 Assessment of assay performance

This assay was performed using a semi-automated SPE sample preparation procedure with detection using a Xevo® TQS tandem mass spectrometer. The desired levels of sensitivity were achieved using this instrument, with an LOQ of 42 pmol/L attained with an S/N ratio greater than 10:1 (peak-to-peak) on 10 occasions (Figure 3.13). The LOD was determined to be 27 pmol/L with linearity at concentrations between 42 and 4,161 pmol/L ($r^2 > 0.996$; correlation coefficient).

The Cortecs column demonstrated suitable chromatographic selectivity, facilitating the separation of aldosterone from its metabolic precursor 18-hydroxycorticosterone with a retention time of 2.22 minutes (*personal communication; Mr. Dominic Foley, Waters UK*). LC-MS/MS analysis time was 4.7 minutes.

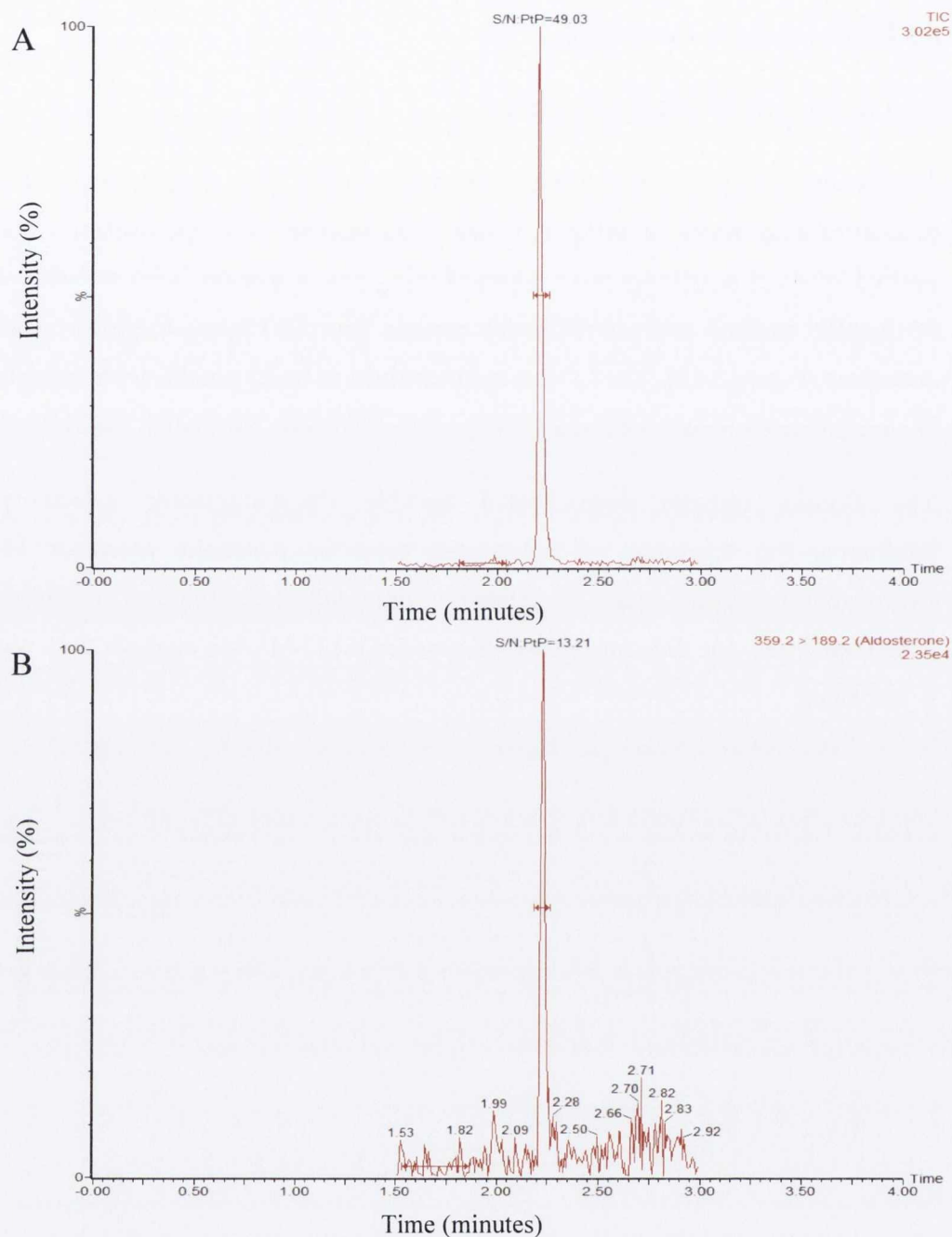


Figure 3.13 Assay procedure 2 S/N values and signal intensity values for TIC (A) and quantifier (B) ion transitions (m/z 359.2>189.2) for plasma QC of 42 pmol/L aldosterone. Retention time; 2.22 min. S/N values were calculated using peak-to-peak assessment as highlighted above with the red arrows.

3.5.2 Assessment of sample suitability and minimum sample volume

In an assessment of suitable sample types for the quantification of aldosterone, there was significant correlation between the paired plasma and serum samples collected (number of paired samples, $n=39$; number of samples, $n=78$) as demonstrated by Pearson product moment correlation coefficient ($r^2=0.9877$; **** $p<0.0001$; 95% CI 0.9882 to 0.9968; Figure 3.14).

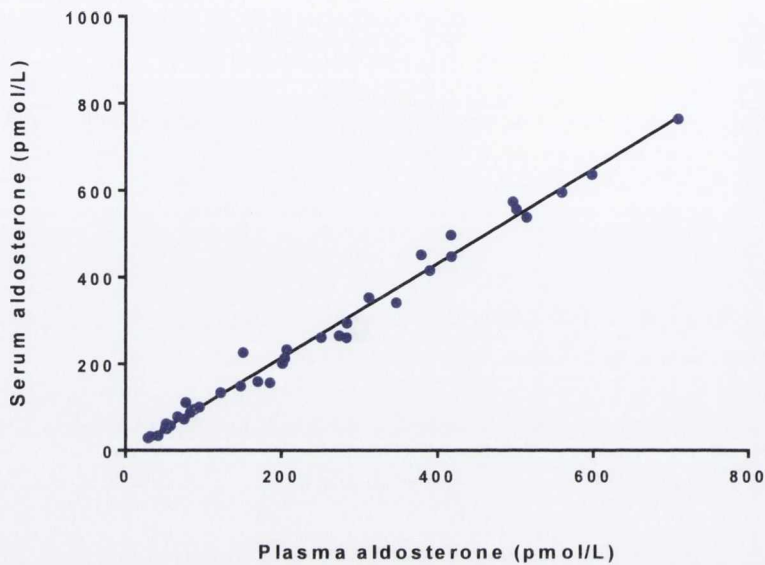


Figure 3.14 Correlation of paired serum and plasma samples in the quantification of aldosterone (assay procedure 2). Comparable measures were obtained using either plasma or serum samples obtained from patients. Total number of patients, $n=39$; total number of samples, $n=78$. $r^2=0.9877$; 95% CI 0.9882 to 0.9968; **** $p<0.0001$; Pearson product moment correlation coefficient (two-tailed correlation).

As sample volume collected from patients is often at a premium, the minimum volume acceptable for analysis was investigated. A QC sample (480 pmol/L aldosterone) was assessed in triplicate at 200, 220, 250 and 300 μ L volumes. The minimum sample volume of 200 μ L evaluated in this study demonstrated suitable precision for assessment of patient samples with a % coefficient of variation (CV) of below 5%.

3.5.3 Evaluation of assay performance, precision, specificity and interference

Using three QC samples assessed in duplicate over a 5-day period, total intra-assay precision was determined to be 9.8%, 7.1% and 4.8% at concentrations of aldosterone of 99, 500 and 2,000 pmol/L.

The accuracy of the assay determined by duplicate assessment of 35 external QC (UKNEQAS) samples over a 5-day period demonstrated a mean negative bias of 23.1% using Bland-Altman plots compared with all method mean values (95% CI -28.6 to -17.6). In a comparison with mean values obtained from all methods, a Deming regression of $y=0.81x - 0.32$ was achieved with a linear regression of $r^2=0.95$. Comparison of assay performance with another LC-MS/MS assay using 59 samples demonstrated a negative bias of -4.9% for this method by Bland-Altman assessment ($p>0.05$, 95% CI -10.3 to 0.5%). Mean recovery in QC samples at concentrations of 100, 500 and 2,000 pmol/L was determined to be 53% (range: 52-55%).

In an assessment of potential steroidal effects on aldosterone quantification, no significant interference was detected from an evaluation of nine steroids as outlined in section 3.3.6. No significant ion suppression or enhancement was detected, with mean net matrix effects of 1.0 (range: 0.9-1.10) and a CV of 7.7% determined using the analyte:internal standard response ratio.

3.5.4 Evaluation of assay accuracy during oncology patient sample assessment

Assay precision was evaluated during the analysis of oncology samples collected for the assessment of aldosterone levels. Three quality control samples with target values of 99 pmol/L, 480 pmol/L and 1,855 pmol/L were assessed in duplicate on seven occasions throughout the assessment of the oncology samples (number of QC samples, n=42). The mean values obtained following assessment were 96 pmol/L $\pm$ 8.1 (range, 84-111 pmol/L), 465 pmol/L $\pm$ 26.9 (range, 426-519 pmol/L) and 1,853 pmol/L $\pm$ 106 (range, 1,600-1,996 pmol/L).

In a comparison of this method with other mass spectrometry assays for aldosterone (number of assays, n=8) during the analysis of oncology patient samples, six UKNEQAS external quality control samples were assessed for initial evaluation of assay performance. This assay demonstrated values which were within 1.5 SD of the mass spectrometry-derived mean for five samples analysed, while one sample was within 2 SD.

3.6 Discussion

LC-MS/MS has emerged as a robust and efficient means of quantifying analytes of interest in the laboratory, demonstrating noteworthy benefit over radioimmunoassay and immunoassay techniques (Adaway *et al.*, 2015). In this study, an LC-MS/MS assay for aldosterone was developed which achieved the levels of sensitivity and specificity required for the assessment of patient samples in clinical practice. An efficient and specific means of sample preparation using a semi-automated SPE procedure was performed demonstrating greater signal intensity and S/N values when compared with other preparation techniques. The automation of this protocol afforded the potential to improve assay precision, without the need for hazardous extraction solvents. Owing to sub-optimal instrument sensitivity, the mass spectrometer initially investigated (Xevo® TQ) failed to quantify aldosterone at low concentrations which may be expected in patient samples. As a result, a more sensitive instrument was utilised (Xevo® TQS) to achieve the desired levels of sensitivity across the clinically relevant concentration range. An LC-MS/MS analysis time of four minutes facilitated a rapid means of assessment with smaller sample volumes required than those necessary for other methods (Hinchliffe *et al.*, 2013; Ray *et al.*, 2014; Meunier *et al.*, 2015), which may be critical where sample volume is at a premium. This assay offers significant benefit to the quantification of aldosterone in the oncology cohort of this study, with additional translational capability into routine clinical practice.

Sample preparation is a crucial component of LC-MS/MS analysis, facilitating the removal of unwanted proteins and interferences from samples which can compromise assay sensitivity. The evaluation of sample preparation techniques in this study demonstrated the superior potential of a semi-automated SPE protocol whilst highlighting significant disadvantages to the use of LLE and SLE in practice. LLE was found to be a time-consuming and difficult procedure to replicate with precision in this study, owing to the need to manually decant solvent from the frozen aqueous component of the sample which may not lead to accurate removal. These findings were in addition to a number of significant disadvantages reported in the literature. Samples containing a high proportion of

surfactant-like compounds which include triglycerides and proteins are noted to potentially form an emulsion between the aqueous and solvent phases (Ramesh *et al.*, 2015) which can accumulate the analyte of interest and prevent its removal when the solvent is decanted. In addition, LLE is challenged by the limited potential for automation which diminishes its translational utility in routine clinical practice (Keevil, 2013). However, despite the outlined potential disadvantages to its use, Ray and colleagues have previously promoted this sample preparation technique as a suitable means of quantifying aldosterone (Ray *et al.*, 2014), however the method incorporated a 2-D chromatographic separation procedure which contrasts with the 1-D technique (utilising one column) applied in this study. While 2-D chromatographic separation is often recognised to facilitate greater peak capacity (Stoll *et al.*, 2007), it incorporates a more elaborate gradient elution process for chromatographic separation and a 2-column configuration which adds complexity to analysis using LLE. The operation of a two-column-based procedure in the study by Ray and colleagues may likely reflect the attempt to compensate for less efficient sample preparation using LLE. Furthermore, the method required a larger sample volume than this assay (250 μ L versus 200 μ L) which in the case of oncology patients may be critical, as small sample volumes were often a feature in this cohort.

SLE was shown in this study to have faster sample preparation times when compared with LLE, however the use of DCM or MTBE as extraction solvents was a significant disadvantage to its use. By virtue of their volatility, the elution of aldosterone using these solvents necessitated the use of a fume hood to eliminate solvent vapour and prevented full automation using the Tecan in the laboratory. This has been regarded as a potential disadvantage to translation into routine clinical use (Keevil, 2013). The manual addition of extraction solvents in SLE, as with LLE may give rise to human errors which are suggested to be greater for LC-MS/MS than those acquired using clinical chemistry analysers (Vogesser & Seger, 2010). Such issues may consequentially affect assay accuracy and precision, thus diminishing their clinical utility in practice. In this study, an S/N ratio of 12:1 was achieved for a pooled, redundant serum sample spiked with 111 pmol/L aldosterone. While comparable the signal:noise ratio reported by Owen

and colleagues using a Waters Xevo® TQ mass spectrometer (11:1) (Owen & Keevil, 2013), it was acknowledged that a constitutive level of aldosterone of 225 pmol/L was present in the pooled serum sample used in this study prior to spiking with 111 pmol/L aldosterone. As such, the S/N value attained from this study was relative to a sample of over 300 pmol/L, which suggests that the optimum S/N ratio would not be achieved at concentrations below 300 pmol/L, thus potentially diminishing the utility of this sample preparation technique.

The SPE protocol performed in assay procedure 2 demonstrated significant improvements in signal intensity and S/N ratio when compared with the SPE technique evaluated in assay procedure 1, highlighting superior potential as a sample preparation technique when compared with LLE and SLE. A notable issue with the SPE procedure employed in assay procedure 1 was the obstruction of wells of the SPE plate following sample loading which prevented subsequent wash and elution steps. This was likely due to incomplete protein precipitation, namely with the absence of a protein precipitation step using zinc sulphate for example. Zinc sulphate has been regarded as one of the superior precipitants for the efficient removal of proteins present in human samples (Polson *et al.*, 2003) and its notable absence in assay procedure 1 likely resulted in direct obstruction of the wells by proteins and other sample components not removed prior to sample loading. The addition of a protein precipitation step with zinc sulphate in the SPE protocol of assay procedure 2 circumvented this issue, facilitating efficient disruption of protein binding with subsequent removal prior to the elution of aldosterone. The SPE extraction technique was developed on the principle mode of ion exchange, whereby aldosterone present in the sample binds to the MAX µelution plate via a hydrophobic mechanism. The wash steps used following sample loading facilitated efficient removal of residual polar analytes and weakly retained analytes, exemplified in the minimal influence of matrix effects when aldosterone was eluted and analysed. While the recovery values for this assay were lower than what would be desired (mean of 53%), the addition of the internal standard prior to SPE sample preparation compensates for the poor recovery achieved as the recovery of the internal standard will match that of the sample being assessed. This minimised the potential effect of sub-optimal

recovery whilst also performing an invaluable role in the detection of interferences (Annesley, 2003). However, as outlined by Annesley, internal standard assessment should not be solely relied upon to detect interferences and other techniques such as the assessment of the qualifier/quantifier ion ratio should be assessed, which was consistent in this study during analysis. Furthermore, no significant steroidal influence or evidence of ion suppression or enhancement were detected, highlighting the efficiency of sample preparation.

SPE has demonstrated significant utility previously where Hinchliffe and colleagues developed an SPE procedure using HLB (hydrophilic-lipophilic balance) plates based on the SPE mode of reversed phase extraction (Hinchliffe *et al.*, 2013). The method by Hinchliffe required a larger sample volume of 250 μL when compared with this study which was a significant disadvantage to its use, as small sample volumes ($<250 \mu\text{L}$) were frequently collected from the oncology patients in this study. Such sample preparation techniques also demonstrate clinical utility by avoiding the need for radioactive materials which is of significant benefit when compared with radioimmunoassay procedures.

A distinct benefit of SPE in this study was the ability to automate the protocol providing high-throughput sample preparation for LC-MS/MS analysis. Furthermore, this mode of preparation reduces the potential for human error by providing an accurate means of dispensing sample and solvent volumes, thus improving the precision of the assay as remarked previously when developing an LC-MS/MS assay (Dunlop *et al.*, 2014). This aspect reinforces the clinical utility of this sample preparation technique, favouring its adoption in routine practice.

The Waters Cortecs® C₁₈ column investigated in this study demonstrated considerable efficacy for aldosterone analysis when utilised with the Waters Acquity UPLC I-Class system and Xevo TQS. The Waters Acquity UPLC® I-class system is recognised to generate low levels of dispersion, also known as extra-column band spreading which when combined with the Cortecs column potentiate the efficiency of the separation. The Cortecs column utilised in this study comprised particles less than 2 μm in size in the packing material, which is recognised to improve separation efficiency in generating sharper peaks when

compared with columns with larger particle sizes. The use of mass spectrometry grade water and methanol as mobile phases A and B respectively in a gradient elution provided an efficient and specific means of chromatographic separation with no significant interference from isobaric compounds or from steroids and with no evidence of substantial ion suppression or enhancement. This contrasted with the potential risk of poor specificity which has been previously associated with immunoassays (Tate & Ward, 2004). This was complemented by a short chromatographic analysis-time of less than five minutes, affording the opportunity for high-throughput analysis. In addition, the relatively small injection volume (25 μ L) utilised in this assay affords the opportunity to re-inject samples if required owing to the 90 μ L sample volume of the eluted sample, which was a demonstrable benefit in cases where re-assessment may be required. The Cortecs column could not be evaluated with the Xevo® TQ as a result of the higher backpressures generated by the column which cannot be accommodated by the Acquity UPLC classic system coupled with the Xevo® TQ in this study. While the PFP column had been reported to demonstrate suitable selectivity with minimal interference (Hinchliffe *et al.*, 2013), this column did not provide the desired levels of sensitivity with the Xevo® TQ, with mobile phase additives not offering improvement in sensitivity or in the signal:noise ratio generated. Owing to the potentially superior chromatographic separation efficiency associated with the Cortecs column, the PFP was not utilised in assay procedure 2.

The Xevo® TQ utilised in assay procedure 1 coupled with the sample preparation procedures assessed (see the discussion of LLE and SLE using the Xevo® TQ) failed to demonstrate the levels of sensitivity required to quantify aldosterone at concentrations near 100 pmol/L which could be expected in clinical practice. This finding prompted development of a suitable assay using the Xevo® TQS which is acknowledged to have greater sensitivity than the Xevo® TQ (assay procedure 2). The Xevo® TQS demonstrated acceptable levels of sensitivity for the quantification of aldosterone across the clinically relevant range with an LOQ of 42 pmol/L at an S/N ratio of greater than 10:1. This was comparable with LOQ values reported elsewhere using the Xevo® TQS (30 pmol/L) (Hinchliffe *et al.*, 2013) as well as using other mass spectrometers (30 pmol/L) (Turpeinen *et al.*,

2008) and (50 pmol/L) (Van Der Gugten *et al.*, 2012). This assay provided suitable levels of precision across the determined range of 42 to 4,161 pmol/L, with the assessment of quality control samples from across the clinical range with overall % CV values of less than 9%. While no National Institute of Standards and Technology (NIST) traceable standard was available for the preparation of aldosterone calibrators or QC to permit the most accurate means of determining aldosterone concentration, the assessment of external quality control (UKNEQAS) samples served as an alternative means of assessing the accuracy of the assay. While this assay demonstrated a negative bias following assessment of 35 UKNEQAS samples when compared with the all method mean values which includes techniques such as immunoassay known to be subject to interferences which could falsely elevate the concentration of aldosterone present in the sample. The findings from this study were similar to those reported for an aldosterone assay developed by Hinchliffe and colleagues where a mean negative bias from the all method mean was identified following NEQAS sample analysis (Hinchliffe *et al.*, 2013).

In a separate assessment of six NEQAS samples directly compared with other mass spectrometry assays for aldosterone during the analysis of oncology patient samples, values were found to be similar with values less than 1.5 SD from the mean values determined by other mass spectrometry assays. The differences observed in this assessment may be attributable to the small number of samples examined and the low number of mass spectrometry assays being compared. However, these limited findings were similar to a method comparison performed with another LC-MS/MS assay whereby the bias between methods was acceptable (<5%), emphasising the comparability of assay performance with other mass spectrometry-derived methods.

This assay has demonstrated significant clinical utility in promoting an efficient means of assessment of aldosterone levels in oncology patients in this study (see Chapter 5). Furthermore, this assay should be favoured for routine analysis over conventional radioimmunoassay and immunoassay methods based on effective sample preparation, rapid LC-MS/MS analysis times and levels of sensitivity and specificity not always feasible with current techniques.

In summary, this study has demonstrated that the robust, semi-automated SPE method evaluated in assay procedure 2, combined with the chromatographic separation capability of the Cortecs C₁₈ column has provided an efficient means of sample preparation. While the Xevo TQ did not demonstrate the necessary levels of sensitivity required for the quantification of aldosterone, this assay would be translatable into routine clinical practice when a Xevo TQS is utilised.

Chapter 4

Evaluation of hs-cTnI in the detection of subclinical anthracycline-mediated cardiac myocyte injury

4.1. Introduction

It is recognised that anthracycline-mediated cardiac injury which often develops in the early stages following treatment initiation (Cardinale *et al.*, 2015; Wang *et al.*, 2015) is commonly reversible with appropriate medical intervention, while cardiac injury presenting following treatment completion is largely irreversible. This emphasises that early detection is essential to allow for the prevention or attenuation of cardiac damage (Vejpangsa & Yeh, 2014). These clinical scenarios present challenges which draw upon the expertise of both cardiology and oncology specialities to offer an effective means of cardiac monitoring during chemotherapy (Patane, 2015). The serial assessment of hs-cTnI offers a potential means of identifying early cardiac myocyte injury in these cases since ECHO has limited sensitivity in detecting subtle subclinical changes.

Since cTnI has good specificity in identifying cardiac myocyte damage, it has been investigated for its potential in detecting and assessing risk of anthracycline-mediated cardiac injury (Sawaya *et al.*, 2011; Ky *et al.*, 2014; Moreno *et al.*, 2014). Early studies of such toxicity among patients treated with chemotherapeutic agents, including anthracyclines, utilised conventional cTnI assays which have provided compelling indications of a correlation between elevated cTnI levels and cardiac impairment (Cardinale *et al.*, 2004). This association has been reinforced with evidence of decreases in LVEF following anthracycline chemotherapy (Specchia *et al.*, 2005). Elevated cTnI using “ultrasensitive” assays and a strong association with peak systolic longitudinal myocardial strain have also been recognised among patients treated with anthracycline-containing regimen, who later developed cardiac injury.

In this study the clinical utility of the novel cardiac biomarker hs-cTnI was investigated, with an LOD of as low as 1.2 ng/L, in the detection of anthracycline-mediated subclinical cardiac myocyte damage in a patient cohort naïve to prior chemotherapy. Furthermore, its value as a monitoring tool in guiding patient management was also evaluated.

The aims of this study were:

- To evaluate hs-cTnI in the detection of anthracycline-mediated subclinical cardiac myocyte injury in an oncology cohort naïve to prior chemotherapy
- To assess the value of hs-cTnI as a monitoring tool in guiding patient management in oncology

4.2 Methods

The 84 patients recruited to this study were classified into two treatment cohorts, based on whether they received an anthracycline-based (number of patients, n=38; Figure 4.1) or a non-anthracycline-based (number of patients, n=46; Figure 4.2) chemotherapeutic regimen. Patients in the anthracycline cohort were further grouped according to the cycles in which the anthracycline agent was administered. Patients who received an anthracycline agent in the first half of their chemotherapeutic regimen (**ACT/ACTH**, cycles 1-4: doxorubicin, cyclophosphamide; cycles 5-8: paclitaxel ± trastuzumab) were assigned to the early-dose group, while patients who received an anthracycline agent in the second half of the regimen (**T-FAC**, cycles 1-4: paclitaxel; cycles 5-8: fluorouracil, doxorubicin, cyclophosphamide) were assigned to the late-dose group. Patients who received an anthracycline agent continuously during their regimen (**R-CHOP**, **CHEOP**, **ABVD**, **DA 3+10** and **doxorubicin** sole agent) were assigned to the continuous-dose group. Doxorubicin (Adriamycin) was the main anthracycline agent used with one patient receiving daunorubicin. Dosing characteristics are described in Table 4.1. A diverse array of chemotherapeutic strategies was utilised in this study, with regimens consisting of between four to eight treatment cycles.

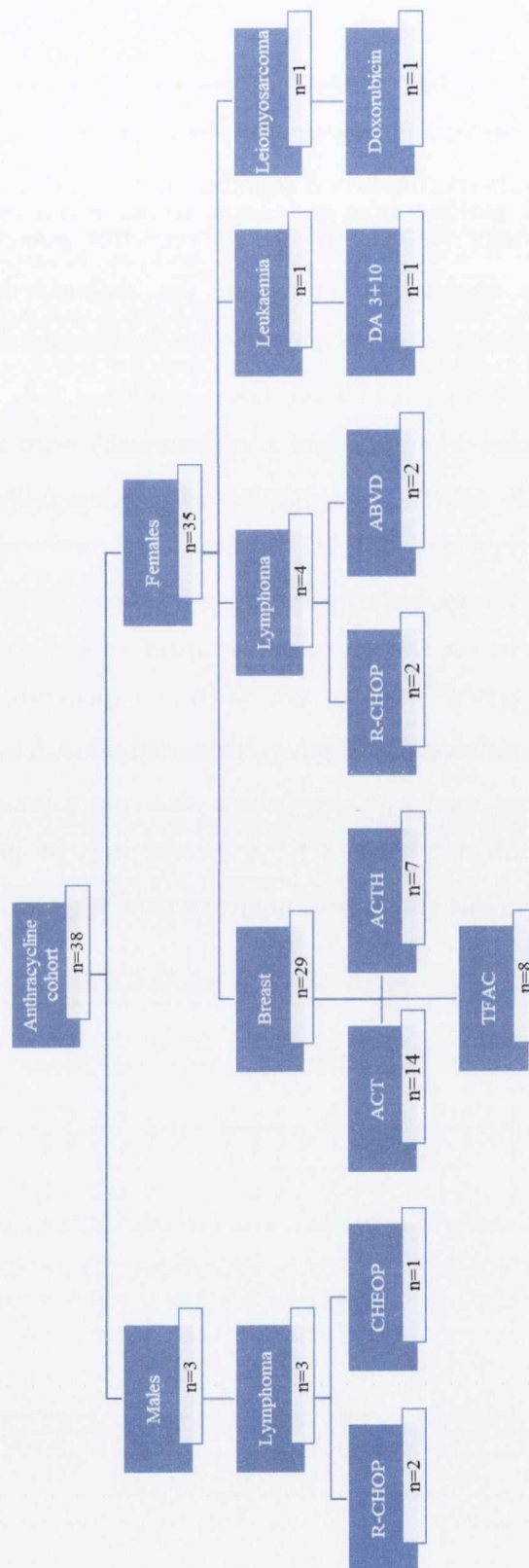


Figure 4.1 Diagnostic and therapeutic characteristics of the anthracycline cohort.

Patients receiving ACT or ACTH regimens were assigned to the early-dose group and patients receiving TFAC were assigned to the late-dose group. Patients receiving all other regimens were assigned to the continuous-dose group. **ABVD**, doxorubicin, bleomycin, vinblastine, dacarbazine; **ACT**, doxorubicin, cyclophosphamide, paclitaxel; **ACTH**, doxorubicin, cyclophosphamide, paclitaxel, trastuzumab; **CHOEP**, cyclophosphamide, doxorubicin, vincristine, etoposide, prednisolone, ranitidine; **DA 3+10**, daunorubicin, cytarabine; **R-CHOP**, rituximab, vincristine, doxorubicin, cyclophosphamide, prednisolone; **T-FAC**, paclitaxel, fluorouracil, cyclophosphamide, doxorubicin.

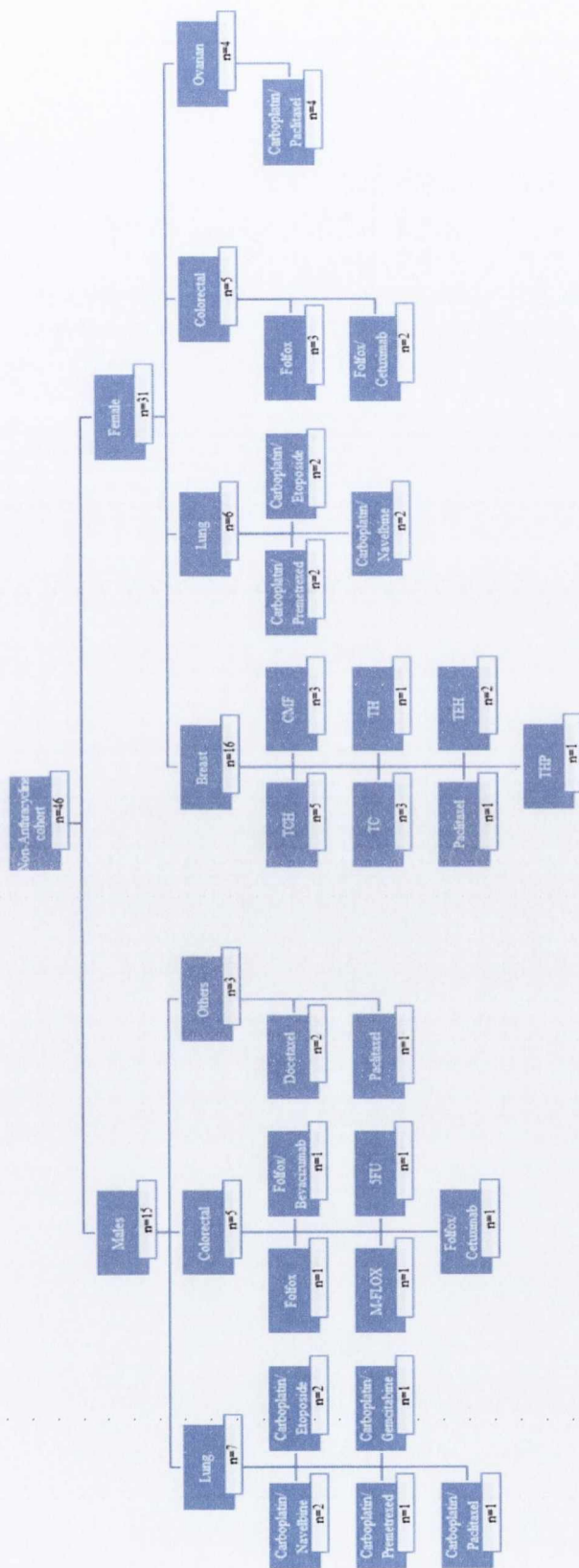


Figure 4.2 Diagnostic and therapeutic characteristics of the non-anthracycline cohort.

Patients receiving chemotherapeutic regimen not containing an anthracycline agent were assigned to the non-anthracycline cohort. These regimen included a diverse array of chemotherapeutic strategies, consisting of between 4-8 treatment cycles. Males-*Others*: Angiosarcoma (n=1), Prostate (n=2). **CMF**, cyclophosphamide, methotrexate, 5-fluorouracil; **Folfox**, oxaliplatin, calcium leucovorin, fluorouracil; **5-FU**, 5-Fluorouracil; **M-FLOX**, calcium leucovorin, oxaliplatin, 5-fluorouracil; **NTT**, naïve to treatment; **TC**, docetaxel, cyclophosphamide; **TCH**, trastuzumab, carboplatin, docetaxel; **TEH**, docetaxel, cyclophosphamide, trastuzumab; **TH**, paclitaxel, trastuzumab; **THP**, paclitaxel, trastuzumab, pertuzumab.

Table 4.1 Dosing characteristics for doxorubicin in the early-dose, late-dose and continuous-dose groups.

Anthracycline group	No. of cycles of treatment	Median dose per cycle (mg) <i>IQR (mg)</i>	Median cumulative dose received (mg) <i>IQR (mg)</i>	Maximum lifetime cumulative dose (%)
Early-dose	4	100.8 (94.2-108.0)	403.2 (376.8-438.0)	54
Continuous-dose	6-8	85 (79-98)	609 (522.1-680.8)	89
Late-dose	4	82.5 (77-89)	327.0 (302-349.4)	44

A 10 ml serum sample collected in standard serum gel separator tubes (Sarstedt, Germany) was collected from each patient prior to commencing chemotherapy, subsequently on the first day of each chemotherapy cycle (of between 14-28 day duration) and where patients returned for clinic visits post-chemotherapy in order to assess trough levels, resulting in hs-cTnI analyses of 606 individual samples. Samples collected were centrifuged at 3,500 g for 10 minutes at room temperature and the serum was aliquoted and frozen at -70°C until analysis.

Serum aliquots taken from each treatment cycle were defrosted, mixed and centrifuged at 3,500 g for 10 minutes before being analysed using an Abbott Diagnostics Architect STAT two-step immunoassay for hs-cTnI (Abbott Architect i2000sr) (see section 2.1.2.1). All patient ECHO procedures were examined and results of this and routine laboratory data were recorded.

hs-cTnI data was assessed using a unisex 99th percentile (25 ng/L) (McGorrian *et al.*, 2013), together with gender-specific 99th percentiles of 16 ng/L for females and 34 ng/L for males (Shah *et al.*, 2015), as determined in the HUNT study and by Apple and colleagues (Apple *et al.*, 2012; Omland *et al.*, 2015). Median relative and absolute δ changes in hs-cTnI were also determined. Results were tested for normality using the D'Agostino-Pearson omnibus normality test. This resulted in the application of non-parametric methods to all hs-cTnI assessments. A parametric statistical test was applied in the assessment of difference in patient ages between cohorts; unpaired t-test. Results were presented as mean $\pm$ SD. Non-parametric statistical tests were applied in the assessment of hs-cTnI from the oncology cohort; Wilcoxon signed-rank test (paired t-test), Mann-Whitney U test (unpaired t-test), Spearman's rank order correlation (two-tailed correlation) and Friedman test (repeated measures ANOVA). Results were presented as median $\pm$ IQR.

4.3 Results

4.3.1 Characteristics of the anthracycline and non-anthracycline patient cohorts

The anthracycline cohort (number of patients, n=38), (mean age, 49 years; SD, 13) was significantly younger than their non-anthracycline counterparts (number of patients, n=46), (mean age, 59 years; SD, 10.23; ***p<0.001, unpaired t-test). The characteristics for each anthracycline group and the non-anthracycline cohort are described in Table 4.2. No patient had an LVEF of below 50% prior to commencing chemotherapy.

Table 4.2 Demographics for anthracycline and non-anthracycline cohorts.

Patient characteristics	Anthracycline Cohort			Non-anthracycline cohort
	Early-dose group	Late-dose group	Continuous-dose group	
Patient Number	21	8	9	46
Age (years)	50	42	53	59
(SD)	(9.9)	(12.08)	(18.45)	(10.23)
ECHO LVEF (%)*	>55	>55	>60	>60 (n=15)

* An ECHO was performed prior to commencement of the first cycle of anthracycline chemotherapy in the anthracycline cohort and prior to treatment in 15 patients in the non-anthracycline cohort. SD, standard deviation; LVEF, left ventricular ejection fraction. Patient ages were expressed as mean $\pm$ SD.

4.3.2 hs-cTnI evaluation in anthracycline and non-anthracycline cohorts prior to chemotherapy

Prior to treatment, hs-cTnI measures were higher in the non-anthracycline cohort (* $p < 0.05$; Mann-Whitney U test; Figure 4.3). With the exception of one patient (female) with a measurement of 16.9 ng/L prior to treatment in the anthracycline cohort, all results were below both the gender-specific (M, 34 ng/L; F, 16 ng/L) and unisex (25 ng/L) 99th percentiles.

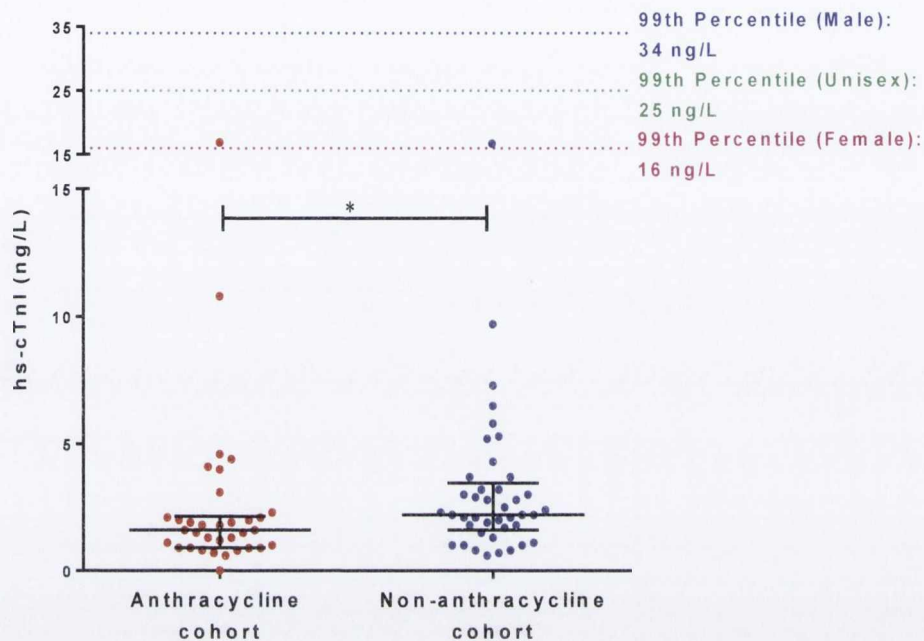


Figure 4.3 Comparison of hs-cTnI concentrations in anthracycline and non-anthracycline cohorts prior to commencing chemotherapy. Significantly higher hs-cTnI measures detected in the non-anthracycline cohort prior to commencement of chemotherapy. Total number of patients, $n=75$; total number of samples, $n=75$. $*p<0.05$; Mann-Whitney U test. Results were expressed as median $\pm$ IQR.

4.3.3 Assessment of hs-cTnI between cycles of chemotherapy

Median hs-cTnI measurements increased significantly in the early-dose group from 1.3 ng/L to 31.2 ng/L following the first five cycles of chemotherapy (number of patients, n=20) (****p<0.0001; Friedman test), with a median relative δ increase of 2,592% and a median absolute δ increase of 30.7 ng/L (Figure 4.4). The greatest relative δ increase in hs-cTnI between cycles of chemotherapy occurred following the first cycle of treatment (median increase, 164%), while the greatest median absolute δ increase occurred between the fourth and fifth cycles of treatment (11.9 ng/L) correlating to the final cycle of anthracycline treatment (with cyclophosphamide) and first cycle of paclitaxel treatment (with trastuzumab in some cases). hs-cTnI concentrations decreased from 31.2 ng/L following five cycles of treatment to 13 ng/L following sixth or seventh cycle of chemotherapy (5-months post-initiation) (number of patients, n=18; *p<0.03; Wilcoxon signed-rank test).

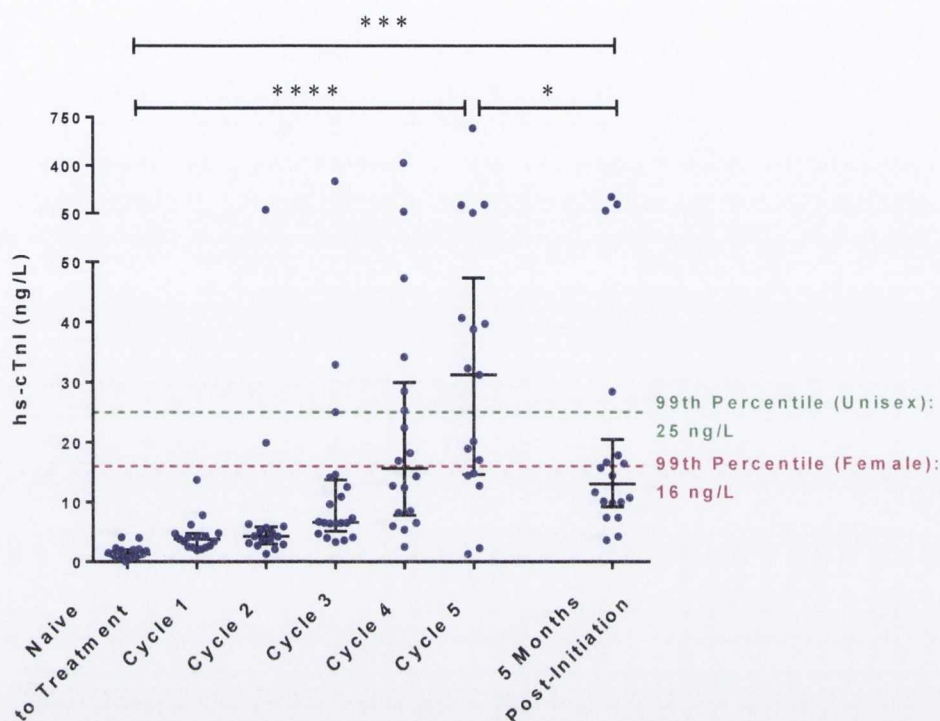


Figure 4.4 hs-cTnI assessment in the early-dose group during each chemotherapeutic cycle. hs-cTnI concentrations increased significantly following the first five cycles of chemotherapy and decreased at assessment five months post-initiation, but remained elevated compared with pre-treatment evaluation. Total number of patients, $n=20$; total number of samples, $n=124$. **** $p<0.0001$; Friedman test (Naïve to treatment-cycle 5). * $p<0.05$; *** $p<0.001$; Wilcoxon signed-rank test (Cycle 5-5 months post-initiation of treatment, naïve to treatment-5 months post-initiation of treatment). Results were expressed as median $\pm$ IQR.

The continuous-dose group demonstrated an increase in median hs-cTnI from 1.75 ng/L to 21.20 ng/L following the fifth cycle of chemotherapy (**** $p < 0.0001$; Friedman test), with a median relative δ increase of 744% and an absolute δ increase of 27.85 ng/L (Figure 4.5). The greatest median relative δ and absolute δ increase in hs-cTnI between cycles occurred between the third and fourth cycles of chemotherapy (128% and 13.7 ng/L respectively).

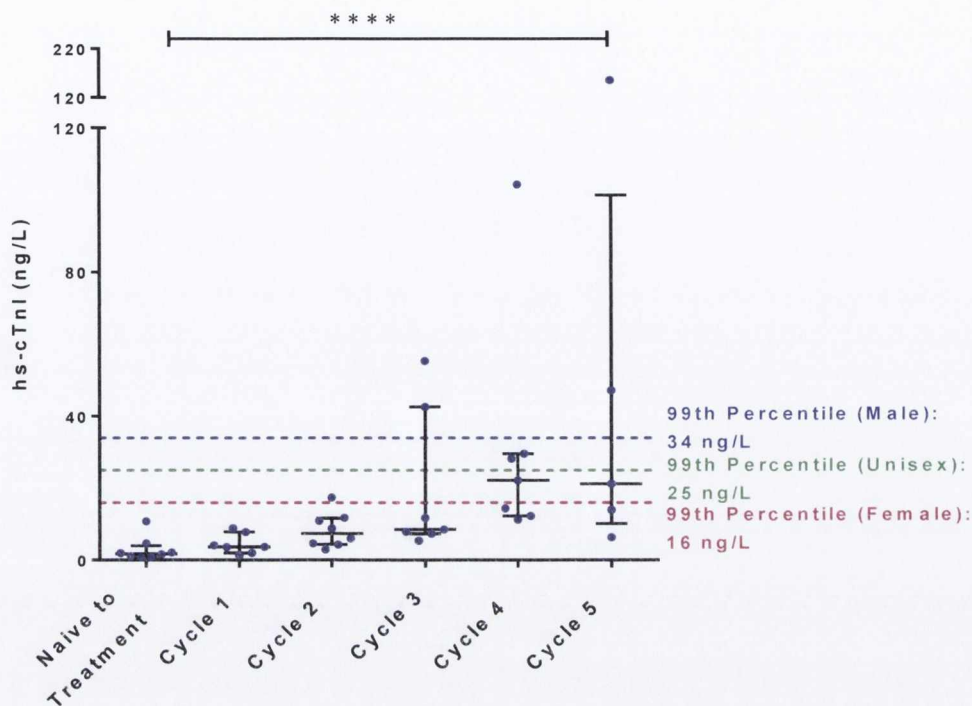


Figure 4.5 hs-cTnI assessment in the continuous-dose anthracycline group during chemotherapy. hs-cTnI increased significantly following the first five cycles of chemotherapy. Total number of patients, n=8; total number of samples, n=44. ****p<0.0001; Friedman test. Results were expressed as median $\pm$ IQR.

The late dose-group demonstrated concentrations of hs-cTnI (Figure 4.6.A) which were comparable with the non-anthracycline cohort (Figure 4.6.B) prior to the introduction of the anthracycline agent, after which a rise was observed. In comparison, hs-cTnI measurements among the non-anthracycline cohort were significantly below the gender-specific and unisex 99th percentiles during all cycles of treatment, with many measurements below the LOD.

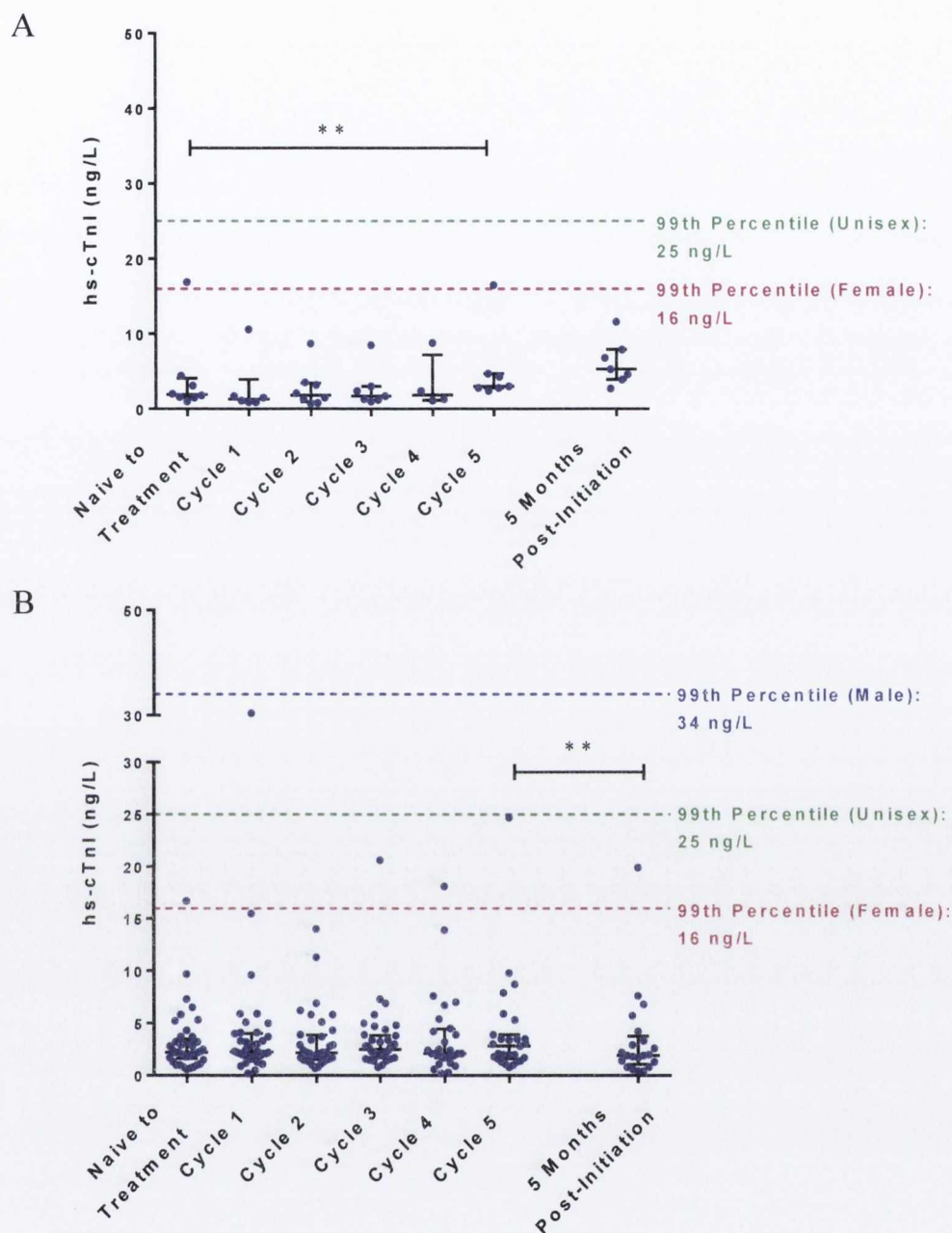


Figure 4.6 hs-cTnI assessment in the late-dose anthracycline group (A) and non-anthracycline cohort (B) during chemotherapy. Significant increase in hs-cTnI following the first five cycles in the late-dose anthracycline cohort, while a later statistically significant decrease was noted in the non-anthracycline cohort. Total number of patients, $n=8$ (A) and 41 (B); total number of samples, $n=47$ (A) and 229 (B). $**p<0.01$; Wilcoxon signed-rank test (Cycle 5 and 5 months post-initiation of treatment). Results were expressed as median $\pm$ IQR.

4.3.4 Comparison of individual patient hs-cTnI measurements at initiation of treatment and following five cycles of chemotherapy

As each anthracycline group demonstrated significant elevations of hs-cTnI following the first five cycles of chemotherapy, individual patient increases were examined. A direct comparison of measures at initiation of treatment with levels measured following the fifth cycle of chemotherapy revealed significant increases in patients in the anthracycline cohort (**** $p < 0.0001$; Wilcoxon signed-rank test; Figure 4.7.A). With one exception, all patients demonstrated increases in hs-cTnI following the fifth cycle. An assessment of individual hs-cTnI levels in patients in the non-anthracycline cohort did not demonstrate significant increases between treatment initiation levels and measures following the fifth cycle of chemotherapy ($p > 0.05$; Wilcoxon signed-rank test; Figure 4.7.B).

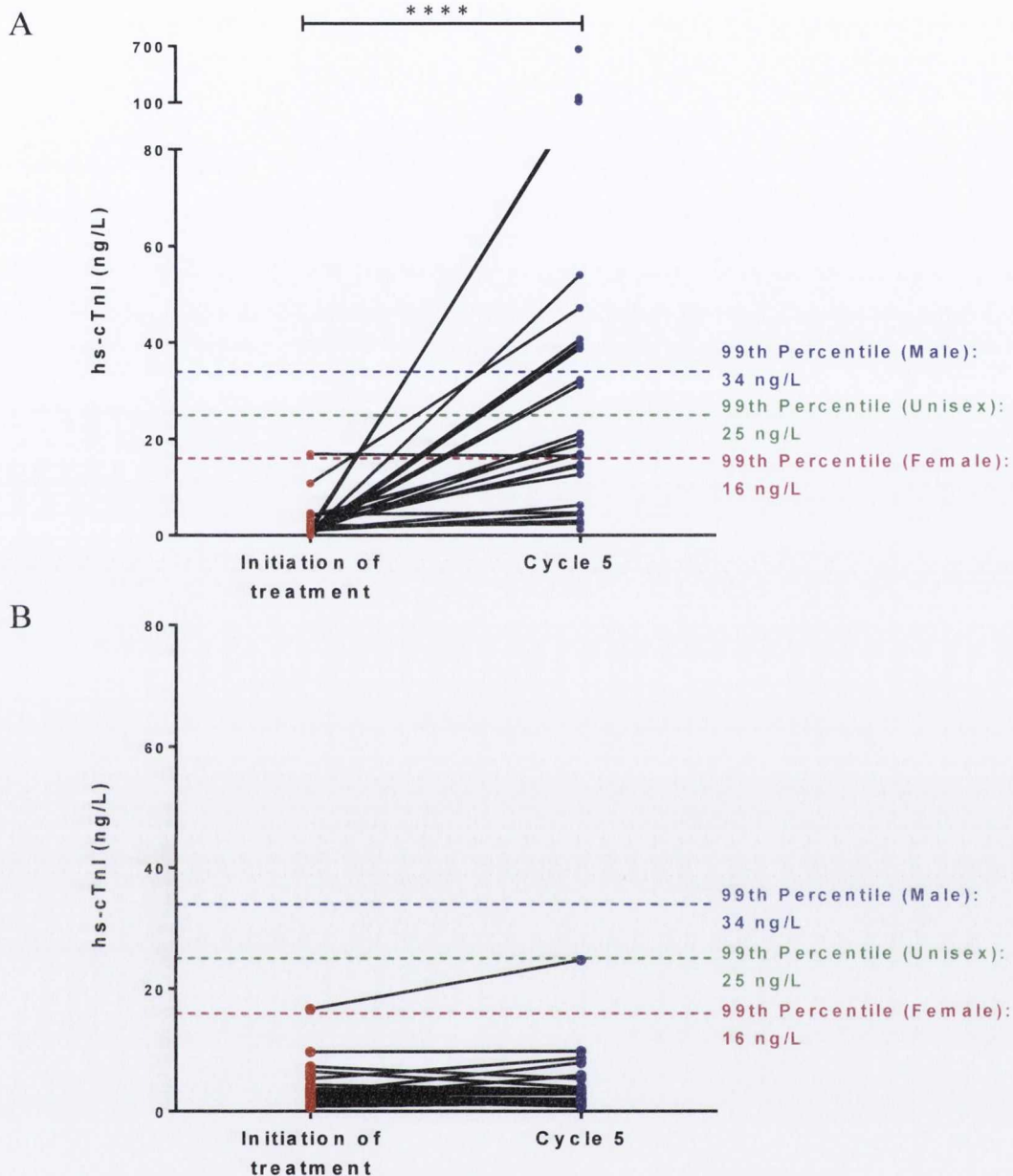


Figure 4.7 Comparison of hs-cTnI measures at initiation of treatment with measures following the fifth cycle in the anthracycline cohort (A) and non-anthracycline cohort (B). Significant elevations in hs-cTnI observed following the fifth cycle of chemotherapy in patients of the anthracycline cohort. Total number of patients, $n=34$ (A) and 41 (B); total number of samples, $n=63$ (A) and 68 (B). **** $p<0.0001$; Wilcoxon signed-rank test. Median results were presented as median $\pm$ IQR.

4.3.5 hs-cTnI measures following completion of chemotherapy

In a small cohort of anthracycline-treated patients (number of patients, n=10), hs-cTnI was reviewed six months post-completion of chemotherapy, revealing an absolute and relative δ decrease of 13.4 ng/L and -171 % from peak treatment measures. While 8 patients demonstrated decreases in hs-cTnI following completion of treatment, with one measurement below the limit of detection on assessment, two patients demonstrated concentrations which had not returned to baseline levels ($p>0.05$; Wilcoxon signed-rank test; Figure 4.8.A). No significant changes were noted in five patients in the non-anthracycline cohort with all hs-cTnI concentrations below 6.5 ng/L ($p>0.05$; Wilcoxon signed-rank test; Figure 4.8.B).

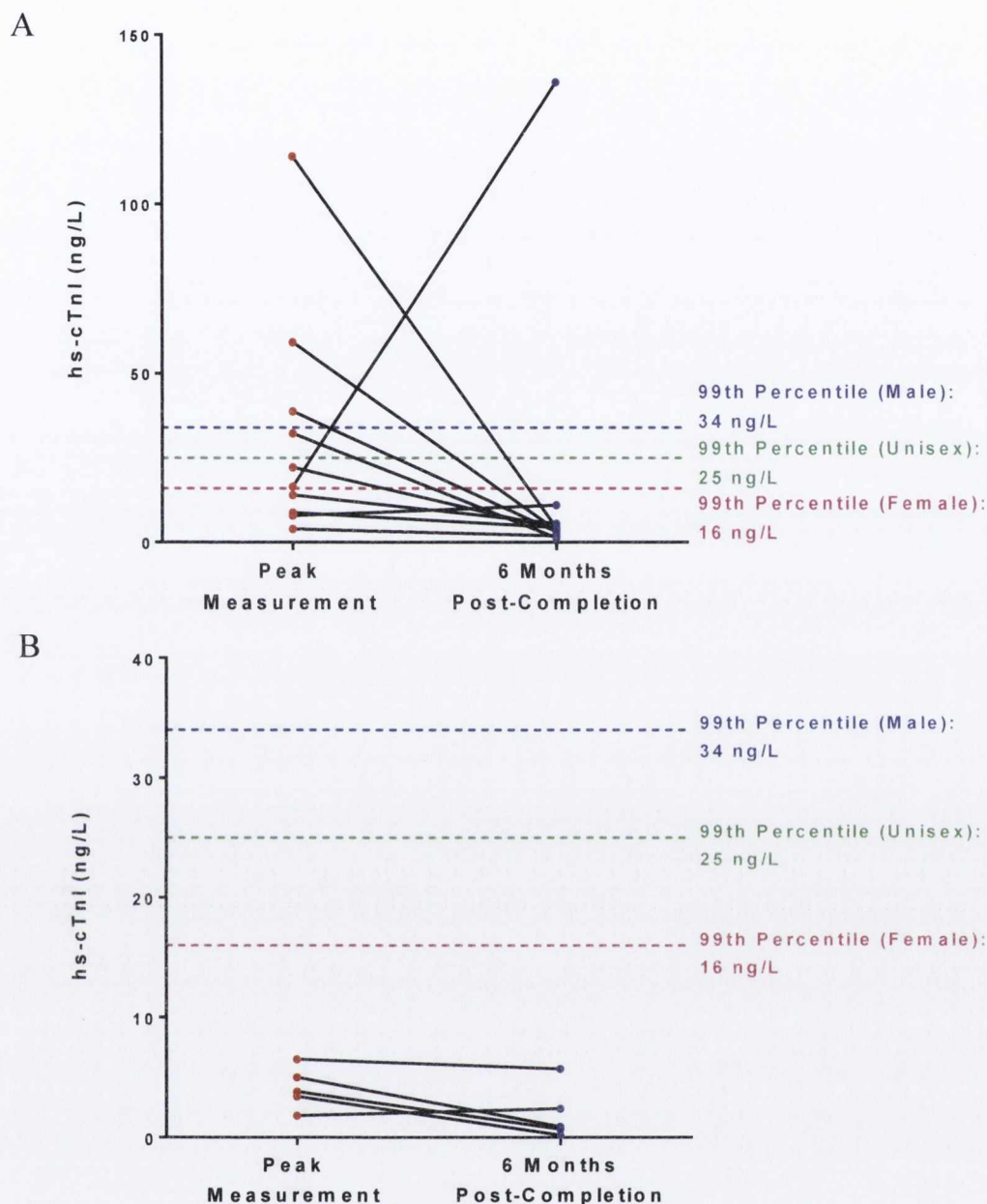


Figure 4.8 Comparison of hs-cTnI concentration at peak measurement compared with six months following completion of chemotherapy in the anthracycline (A) and non-anthracycline (B) cohorts. hs-cTnI predominantly decreased from peak measures when assessed six months following treatment in the anthracycline cohort, whilst concentrations were unchanged in the non-anthracycline cohort. Total number of patients, n=10 (A) and 5 (B); total number of samples, n=20 (A) and 10 (B). $p>0.05$; Wilcoxon signed-rank test.

4.3.6 Comparison of chemotherapeutic dose with hs-cTnI measures

In a direct assessment of doxorubicin dose when compared with hs-cTnI concentration (number of paired samples, $n=123$), no direct correlation was observed ($r = -0.06$; $p > 0.05$; 95% CI -0.23 to 0.13 ; Spearman's rank order correlation; Figure 4.9). Similar findings were also identified in all three anthracycline groups (average across all treatment cycles $r < -0.22$; Spearman's rank order correlation).

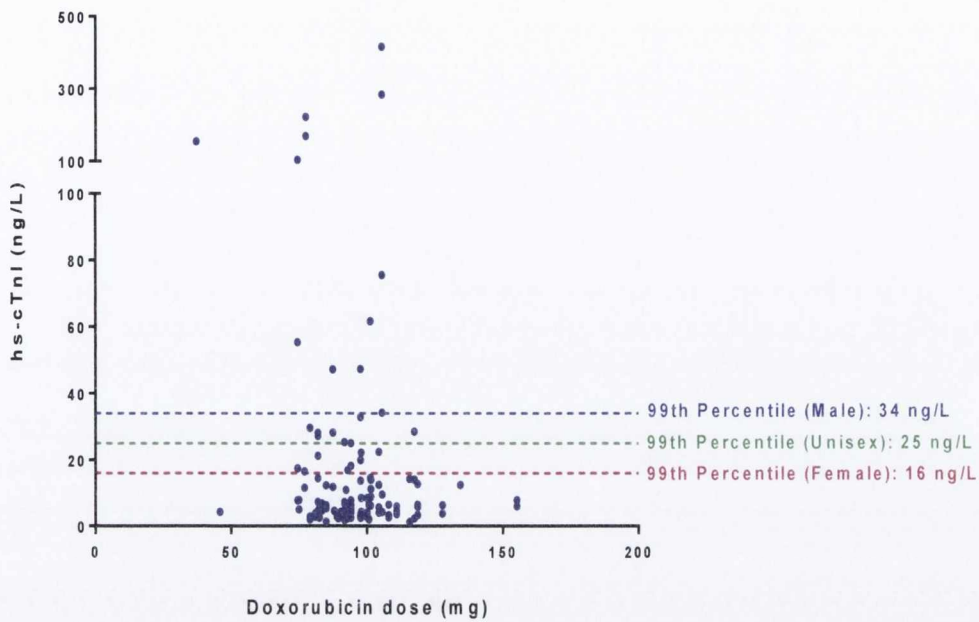


Figure 4.9 Correlation between hs-cTnI concentration and doxorubicin dose per cycle in anthracycline cohort. No correlation between the dose of doxorubicin administered in a cycle and the concentration of hs-cTnI was demonstrated. Total number of patients, $n=38$; total number of samples, $n=123$. $r=-0.06$; 95% CI -0.23 to 0.13 ; $p>0.05$; Spearman's rank order correlation (two-tailed).

In an evaluation of patients receiving cyclophosphamide (maximum dose was $<750 \text{ mg/m}^2$) in the non-anthracycline cohort, no significant increase in hs-cTnI was detected following the first five cycles of chemotherapy ($p>0.05$; Friedman test). Similarly in patients treated with paclitaxel, no significant changes were identified ($p>0.05$; Friedman test). No significant difference in hs-cTnI was observed between patients in the early-dose group who received trastuzumab (number of patients, $n=7$) when compared with those who did not (number of patients, $n=14$), ($p>0.05$; Mann-Whitney U test). Similarly, no significant elevations of hs-cTnI were identified among patients of the non-anthracycline cohort treated with trastuzumab (number of patients, $n=9$), ($p>0.05$; Friedman test).

4.3.7 Assessment of renal function and correlation with hs-cTnI measurements

There is been a growing body of evidence investigating the associations between renal function and hs-cTnI with impairments in renal function, denoted by decreases in eGFR suspected to potentially mediate increases in hs-cTnI, independent of cardiac injury. In an evaluation of eGFR as calculated by CKD-EPI SCr and the corresponding hs-cTnI values from the study cohort during the course of chemotherapy (number of paired measures, $n=458$), no significant correlation between hs-cTnI and eGFR was identified ($r = -0.06$; $p>0.05$; 95% CI -0.15 to 0.04; Spearman's rank order correlation; Figure 4.10). Similar findings were obtained when correlations of eGFR and hs-cTnI were assessed in patients treated with anthracycline agents ($r = -0.06$; $p>0.05$; 95% CI -0.19 to 0.08; Spearman's rank order correlation; Figure 4.11.A) and while regarded as statistically significant, there was also a poor correlation noted in those treated with non-anthracycline agents ($r = -0.32$; $p<0.0001$; 95% CI -0.43 to -0.20; Spearman's rank order correlation; Figure 4.11.B).

A total of five out of 41 measurements greater than the 99th unisex percentile for hs-cTnI (with four of these measures also greater than the gender-specific 99th percentile), all recorded in patients from the anthracycline cohort correlated to an eGFR below $90 \text{ ml/min/1.73m}^2$ which can be indicative of renal impairment.

Furthermore, two of these hs-cTnI values above the unisex 99th percentile corresponded to eGFR values below the 60 ml/min/1.73m² which would typically be associated with acute or chronic kidney diseases (Levey *et al.*, 2015). Both measures, one of which was also above the 99th percentile for males, were obtained from the same patient who presented with an eGFR consistently below 60 ml/min/1.73m² during the course of chemotherapy and was diagnosed with CKD based on prior results.

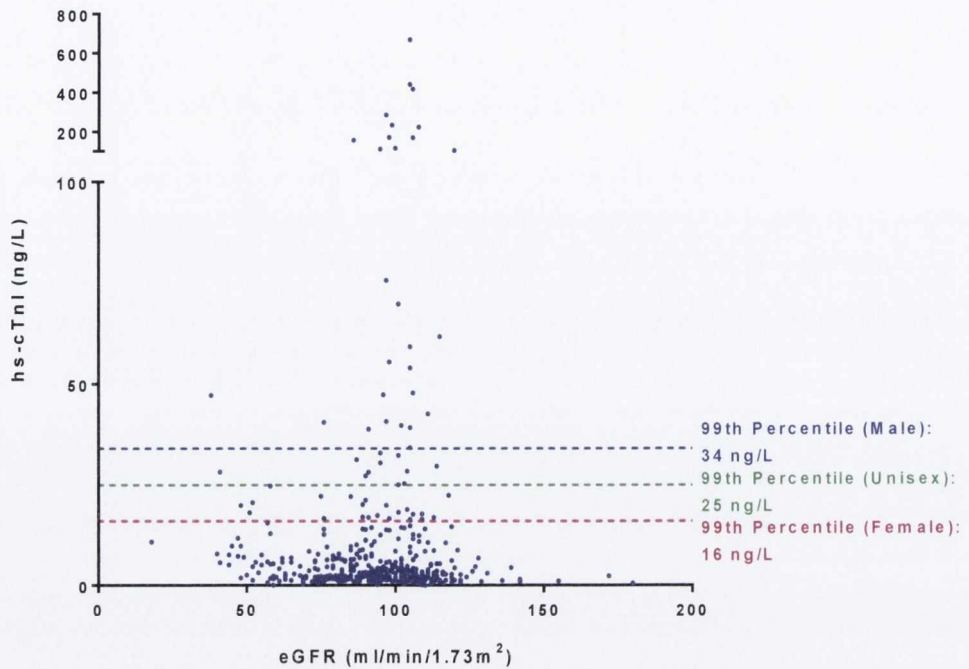


Figure 4.10 Correlation between hs-cTnI concentration and eGFR measurements. No significant correlation demonstrated between hs-cTnI concentration and eGFR measure in the study cohort. Total number of patients, n=84; total number of samples n=458. eGFR was calculated using CKD-EPI SCr (ml/min/1.73m²). $r = -0.06$; 95% CI -0.15 to 0.04; $p > 0.05$; Spearman's rank order correlation (two-tailed).

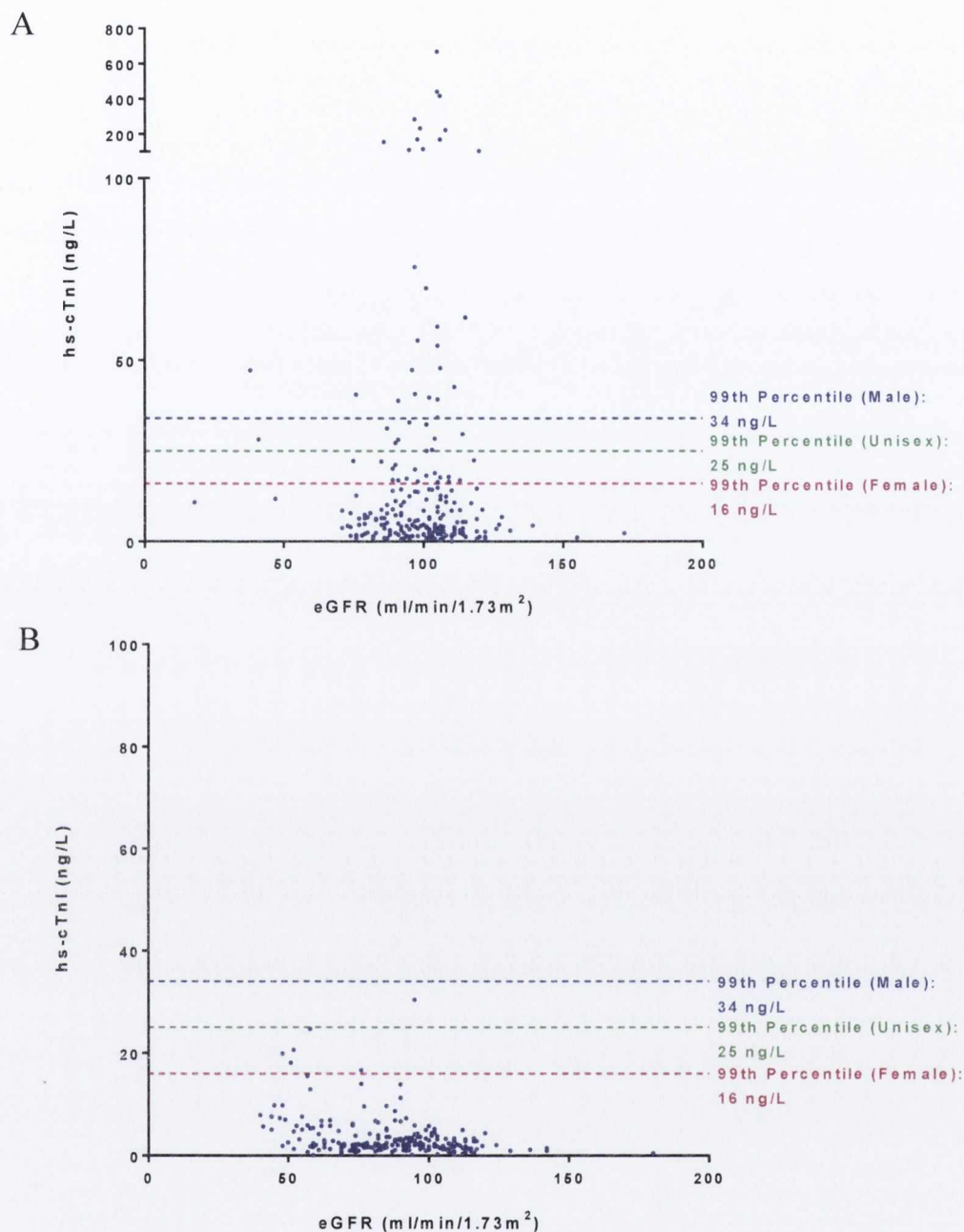


Figure 4.11 Correlation between hs-cTnI concentration and eGFR measurements in the anthracycline (A) and non-anthracycline (B) cohorts.

No significant correlation was demonstrated in patients of the anthracycline and non-anthracycline cohorts. Total number of patients, n=38 (A) and 46 (B); total number of samples, n=222 (A) and 236 (B). eGFR was calculated using CKD-EPI SCr (ml/min/1.73m²). (A) $r = -0.06$; 95% CI -0.19 to 0.08; $p > 0.05$; (B) $r = -0.32$; 95% CI -0.43 to -0.2; $p < 0.0001$; Spearman's rank order correlation (two-tailed).

4.3.8 Assessment of C-reactive protein and white blood cell counts

No patients in the anthracycline or non-anthracycline cohort were found to have significant sepsis, and where C-reactive protein was measured, elevated values were not associated with an elevated hs-cTnI and there was no definitive correlation between hs-cTnI and white cell count ($r = -0.2766$; $p < 0.05$; 95% CI - 0.52 to 0.01; Spearman's rank order correlation; Figure 4.12).

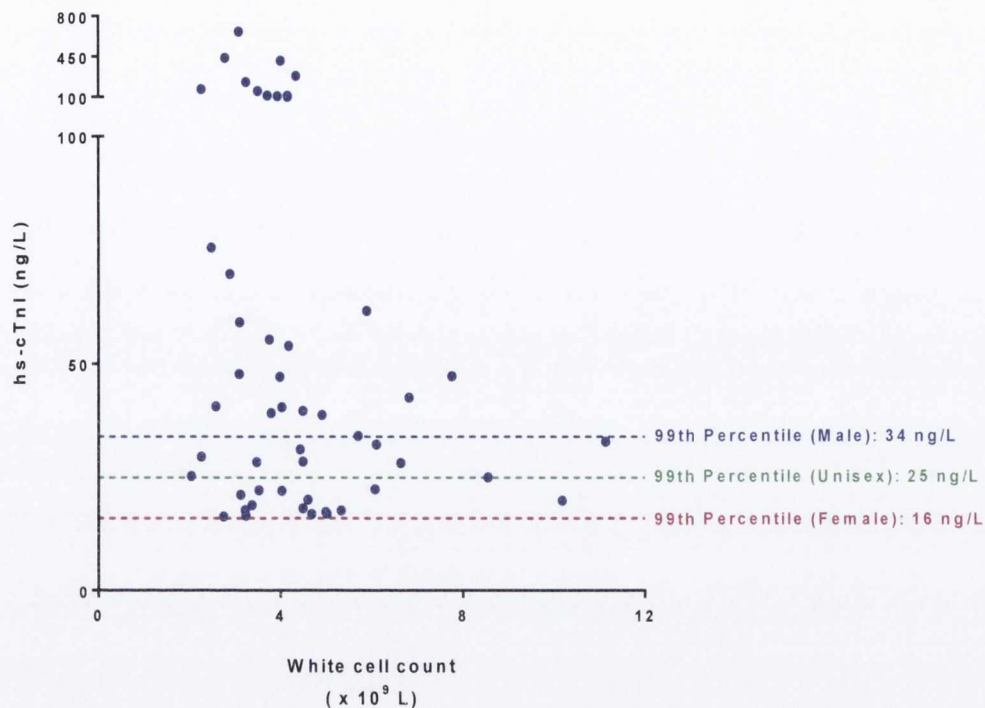


Figure 4.12 Correlation between elevated hs-cTnI concentrations and white cell counts in the anthracycline cohort. No distinct correlation was detected between white cell count level and hs-cTnI concentration. Total number of patients, $n=34$; total number of samples, $n=52$. $r = -0.2766$; 95% CI -0.52 to 0.01 ; $p < 0.05$; Spearman's rank order correlation (two-tailed).

4.3.9 ECHO-based assessment of cardiac injury and correlation to hs-cTnI levels

All patients in the anthracycline cohort (number of patients, n=38) and 21 of the 46 patients in the non-anthracycline cohort had ECHO imaging. Baseline LVEF was higher in the continuous-dose group when compared with early-dose and late-dose groups (see Table 4.2). No patient presented with diastolic or systolic dysfunction prior to commencement of chemotherapy.

Two patients in the anthracycline cohort (early-dose group, both female) demonstrated evidence on ECHO of Grade I diastolic dysfunction which correlated to elevated hs-cTnI levels of 25 ng/L and 28.5 ng/L. Two patients in this cohort also demonstrated decreases in LVEF by 5%, which were preceded by elevations of hs-cTnI to levels above the 99th percentile for females.

Two patients from the anthracycline cohort who demonstrated elevations of hs-cTnI above the 99th percentile for females demonstrated trace tricuspid valve regurgitation (TR) on ECHO assessment. In one case, hs-cTnI concentrations increased following development of TR. Mitral valve regurgitation was detected in three patients in the early-dose group, in which symptoms presented in two cases prior to hs-cTnI elevation.

One patient in the non-anthracycline cohort demonstrated evidence of Grade I diastolic dysfunction (EF of 55-60%) with no increase in hs-cTnI.

4.4 Discussion

It is recognised that there are strong links between cancer and heart disease and a growing clinical need for cardio-oncology patient management. In this era of highly sensitive biomarkers, the application of hs-cTnI in the detection of subclinical cardiac damage during anthracycline chemotherapy has enhanced the ability to assess, monitor and provide recommendations for the care of patients treated with this agent. The specificity and improved sensitivity of hs-cTnI, with an analytical LOD of as low as 1.2 ng/L, offers improved diagnostics in identifying patients at greater risk of cardiotoxicity, over and above what was available previously with conventional cTnI assays, which had analytical functional sensitivity in the region of 30 ng/L. The Roche hs-cTnT with an LOD of 5 ng/L is a commonly used automated cTn assay which also has the potential to be used in the setting of monitoring and follow-up of patients during and post-anthracycline chemotherapy. Early changes are rarely detectable using ECHO with longer follow-up required, which is generally only performed in patients who are symptomatic or those with pre-existing cardiac disease (McKillop *et al.*, 1983).

This study assessed hs-cTnI levels in a cohort of patients with no significant cardiac impairment detectable prior to commencing chemotherapy. With the exception of one patient in the anthracycline cohort who presented with a measure above the 99th percentile for females of 16 ng/L (16.9 ng/L), no hs-cTnI measurement above the 99th percentile (unisex or gender-based) was detected before treatment. While a significant difference between hs-cTnI measures in both cohorts was noted, the difference was merely statistical as such levels of hs-cTnI are not indicative of cardiac damage. Such findings were supported by LVEF assessments which indicate good cardiac function. These initial assessments are of significance as it implicates the toxic effects of anthracycline agents in the observed increases of hs-cTnI during chemotherapy.

hs-cTnI measurements increased significantly following the first five cycles of chemotherapy among our early-dose and continuous-dose groups. These findings correlated with increases in hs-cTnI three months into chemotherapy treatment in

patients treated with doxorubicin-containing regimen (Putt *et al.*, 2015). There was no change in hs-cTnI in patients in the late-dose group prior to the introduction of an anthracycline agent, however a small but significant rise in hs-cTnI was observed thereafter, justifying the conclusion that an assessment of anthracycline-mediated cardiac myocyte injury can be monitored using hs-cTnI. While research has shown an increase in hs-cTnI levels with increasing age (Zeller *et al.*, 2015), the anthracycline cohort was comprised of a younger group of patients which reduces the influence of age, if any, on hs-cTnI elevations observed in this study. As there is no agreed consensus in the serial measurements as to whether relative or absolute δ changes in troponin correlates best with extent of damage, both were reported in these experiments (Apple & Morrow, 2012; Simpson *et al.*, 2014).

Where there were differences in the magnitude of hs-cTnI increases in the three anthracycline-treated groups, there is a direct relationship between anthracycline therapy and sub-clinical myocyte injury as assessed by the pattern of hs-cTnI release. In assessing hs-cTnI data the 99th percentile data are generally referred to for clinical decision-making, however it is of less importance in this setting, than the increased hs-cTnI released as anthracycline chemotherapy progresses. For example, the considerable increase in median relative and absolute δ in hs-cTnI during the first five cycles of treatment among the early-dose group (2,592% and 30.7 ng/L respectively) is indicative of significant myocyte injury, given the awareness that smaller absolute δ increases in hs-cTnI in a non-ACS clinical setting are considered to be significant (Wildi *et al.*, 2015). The greater rises in hs-cTnI were seen in the early-dose group whose maximum cumulative dose of doxorubicin was 53% and median dose per cycle was 101 mg, while the continuous group were administered 89% of maximum dose, characterised by a median dose per cycle of 85 mg. This finding when combined with the greater absolute δ increase in hs-cTnI in the early-dose group suggests that the continuous regimen which utilised smaller doxorubicin doses per cycle resulted in less acute myocyte injury. This finding warrants additional study in larger patient cohorts. It has been acknowledged that toxicity mediated by anthracyclines is not explicitly

dose-dependent which is reaffirmed by the absence of a direct correlation between hs-cTnI and doxorubicin dose in this cohort (Volkova & Russell, 2011).

hs-cTnI was evaluated prior to commencing each cycle of chemotherapy, which provided an assessment of cardiac status the furthest time from the prior cycle (typically between 14-21 days for our anthracycline cohort). Recent literature has suggested that significant cardiac damage leads to troponin release from the troponin complex which persists for a number of days, a theory established in the pathobiology of ACS (White, 2011). This is in comparison to the early releasable pool or cytosolic source of troponin which appears to induce a transient increase in troponin which occurs on the 1st day of injury (Katus *et al.*, 1991). As elevations of hs-cTnI were detected at least 14 days post-administration of the prior cycle of chemotherapeutic agents, these levels would indicate that increases most likely reflect chronic cardiac myocyte injury.

There was no significant cardiac troponin I release observed in patients receiving trastuzumab. Given the extensive literature detailing the cardiotoxic potential of this agent (Keefe, 2002; McArthur & Chia, 2007; Cardinale *et al.*, 2010), these results, albeit in a very small cohort, suggest that cardiac myocyte injury is not a major factor with this treatment at doses less than the maximum administered dose of 8 mg/kg. While most patients of the anthracycline cohort also received cyclophosphamide and paclitaxel, other agents known to cause cardiotoxicity (Senkus & Jassem, 2011), it is not suspected that either agent is solely mediating increases in hs-cTnI, although a synergistic potentiation of cardiotoxicity may be possible. Given the existing knowledge in the literature specifically regarding anthracycline toxicity and the fact that in this study, patients treated with cyclophosphamide, paclitaxel and trastuzumab as part of non-anthracycline regimens (typically of higher doses) did not demonstrate hs-cTnI elevations, this evidence points to anthracyclines as the main cause of cardiac myocyte necrosis and hs-cTnI release in this study. Furthermore, the decrease in hs-cTnI levels from peak measures at assessment 6 months following the completion of chemotherapy in the anthracycline cohort coincides with the completion of anthracycline treatment, which reinforces the concept that this treatment likely induces the observed increases of hs-cTnI.

In the evaluation of hs-cTnI in this study, elevated levels above unisex and gender-specific 99th percentiles were not found to be related to impaired renal function. It has been suggested that elevated measures of cTnI and cTnT can be linked with renal impairment (KB., 2013), possibly in association with some degree of cardiac involvement (Bjurman *et al.*, 2015). However in this study, renal injury did not appear to mediate the observed increases as most hs-cTnI values identified above the 99th percentiles were greater than 90 ml/min/1.73m², conventionally associated with normal renal function. Additionally, infection and sepsis in particular has been associated with cTnI elevation (Ammann *et al.*, 2001). In this study, there was no distinct correlation between white cell counts and hs-cTnI levels or any association between hs-cTnI and CRP which indicated that infection did not significantly influence hs-cTnI measures in this study. As such, this evidence strongly supports the hypothesis that cardiac injury, most likely mediated by anthracycline agents mediates the observed increases in hs-cTnI in this study.

During the course of this study, two patients in the anthracycline cohort were diagnosed with Grade I diastolic dysfunction. As the diagnoses correlated with hs-cTnI levels above 99th percentiles (females and unisex), such findings, albeit in the context of small patient numbers, reinforce the utility of hs-cTnI in the detection of cardiac injury. The poor levels of sensitivity using ECHO to detect subclinical cardiac myocyte injury, as earlier described promote the use of hs-cTnI based on its potential to detect subclinical injury in cardiac myocytes as well as its ability to detect more substantial cardiac damage detected by ECHO as demonstrated in this study.

In this study, normal hs-cTnI measurements were observed among patients treated with non-anthracycline regimens following the first five cycles of chemotherapy and at 6-month follow-up, suggesting the value of serial hs-cTnI monitoring is reserved for patients receiving anthracycline-based therapies.

hs-cTnI or hs-cTnT with highly sensitive levels of detection can be used to risk stratify patients with cardiac injury for close monitoring. While this study was not designed to assess long-term cardiovascular implications of elevated hs-cTnI in

individual patients, the value of this sensitive biomarker has been proven and could be beneficial as a tool for early recognition of patients susceptible to longer term risk of chronic cardiac dysfunction following anthracycline chemotherapy, as has been demonstrated in recent literature (Cardinale *et al.*, 2015).

Anthracyclines are effective anti-cancer agents and the recognition of associated cardiac side effects has encouraged the emergence of cardio-oncology collaborative efforts which has resulted in primary and secondary cardioprotective strategies, including alterations in modes of anthracycline delivery and use of cardioprotective agents, which modulate the RAAS as well as the iron-chelating drug, dexrazoxane (Swain *et al.*, 1997). hs-cTnI has demonstrated significant ability to detect subclinical cardiac injury and may facilitate the assessment of the therapeutic efficacy of such cardioprotective agents and their ability to reduce or prevent cardiac injury. A further benefit of the evidence presented for the use of hs-cTnI is the availability of the test with rapid turnaround as opposed to biomarkers such as myeloperoxidase, growth differentiation factor 15 and placental growth factor shown to improve identification of patients who are at risk for cardiotoxicity, but without widely accessible automated assays (Putt *et al.*, 2015).

Chapter 5

Evaluation of NT-proBNP, galectin-3 and aldosterone in the detection of anthracycline- mediated cardiac injury

5.1. Introduction

Many chemotherapeutic agents are recognised to confer a significant risk of cardiac damage, often observed as cardiac myocyte injury as demonstrated by increases in hs-cTnI in this study. The activation of neurohormonal pathways and manifestation of adverse cardiac remodelling may also ensue following chemotherapy-mediated cardiac damage with the potential to significantly affect cardiac function. As such, early detection is merited to limit the extent of cardiac injury. On account of their association with these pathological mechanisms, the assessment of NT-proBNP, aldosterone and galectin-3 may afford a sensitive and specific means of detecting subclinical cardiac injury mediated by cardiotoxic chemotherapeutic agents.

These biomarkers have demonstrated significant efficacy in the detection of cardiac remodelling and injury, prompting an examination of their role in chemotherapy-mediated cardiac injury. NT-proBNP is released following cardiac stress induced by increased cardiac pressure or volume overload states (Kim & Januzzi, 2011), with persistent elevations of NT-proBNP previously linked with left ventricular impairment following anthracycline chemotherapy (Romano *et al.*, 2011). Aldosterone plays a significant role in blood pressure regulation and in a recent study it was demonstrated that treatment with the aldosterone antagonist spironolactone yielded promising cardioprotective effects in preventing decreases in LVEF during anthracycline chemotherapy (Akpek *et al.*, 2015). Additionally, increased levels of galectin-3 have been associated with left ventricular impairment and myocardial fibrosis (Lepojarvi *et al.*, 2015) and on account of such links, an examination of patients receiving cardiotoxic chemotherapy treatment demonstrated significant associations with cardiotoxicity at subsequent assessment (Putt *et al.*, 2015). The evaluation of these biomarkers may assist in identifying cardiac injury which results in the activation of neurohormonal pathways and initiation of cardiac remodelling.

NT-proBNP, aldosterone and galectin-3 are recognised to confer significant promise in the detection of chemotherapy-mediated cardiac injury prompting their evaluation in this study as a potential means of detecting cardiac injury.

The aims of this study were:

- To investigate the concentration of NT-proBNP during the course of chemotherapy when compared in a cohort of patients receiving anthracycline and non-anthracycline-based regimen
- To evaluate aldosterone levels in the oncology cohort during chemotherapy, with comparison to a normal reference population
- To determine the levels of galectin-3 in a cohort of oncology patients receiving anthracycline-based and non-anthracycline based regimen

5.2. Methods

As previously described in Chapter 4, patients receiving anthracycline-based chemotherapeutic regimen were assigned to the anthracycline cohort (see Figure 4.1) and grouped based on the administration schedule of the anthracycline agent (early-dose, continuous-dose and late-dose anthracycline groups). Doxorubicin (anthracycline) dosing characteristics are detailed in Table 4.1. Patients receiving other chemotherapeutic regimens were assigned to the non-anthracycline cohort for biomarker evaluation (see Figure 4.2). Baseline LVEF was recorded where an ECHO was performed (see Table 4.2).

Serum samples of 10 mL volume were collected from all patients at initiation of and mid-way through the cycles of the chemotherapeutic regimen (mid-treatment measurement) and at final measurement. Samples were centrifuged at 3,500 g for 10 minutes and stored at -70°C prior to analysis.

Aliquots were defrosted, mixed and centrifuged at 3,500 g for 10 minutes before being analysed by an electrochemiluminescence immunoassay for NT-proBNP (Roche cobas e411) with aliquots centrifuged at 11,000 g for five minutes for assessment of galectin-3 using a two-step immunoassay (Abbott Architect i1000sr) (see section 2.1.2.1). Aldosterone was measured using an LC-MS/MS assay developed for this study (see Chapter 3; assay procedure 2) with samples centrifuged at 3,500 g and prepared using an automated (Tecan) SPE procedure prior to analysis.

NT-proBNP concentrations were assessed using a unisex upper reference limit (URL) of 125 pg/mL, adopted in other biomarker studies for the detection of cardiac injury (Sawaya *et al.*, 2012). The URL for aldosterone of 1,314 pmol/L, developed from a reference range study in the Mater Diagnostic laboratory (*Dr. M MacMahon, personal communication*) was utilised as an upper reference limit for assessment. All values below the LOD of the LC-MS/MS aldosterone assay (27 pmol/L) were included in statistical assessments by designation of the LOD value of 27 pmol/L to each result, as previously described (O'Shea *et al.*, 2015). The URL for galectin-3 of 28 ng/mL, established by Gaze and colleagues in a healthy cohort (Gaze *et al.*, 2014) was used to evaluate galectin-3 levels in this study.

Results were tested for normality using the D'Agostino-Pearson omnibus normality test. Non-parametric statistical tests were applied in the assessment of all biomarkers from the oncology cohort; Wilcoxon signed-rank test (paired t-test), Mann-Whitney U test (unpaired t-test), Kruskal-Wallis H test (one-way ANOVA) and Friedman test (repeated measures ANOVA). Results were presented as median $\pm$ IQR.

5.3. Results

The detection of cardiac injury in oncology has traditionally relied upon the assessment of imaging procedures such as ECHO and ECG. However such methods are not always conducive to routine assessment and newer techniques including the evaluation of biomarkers known to be associated with cardiac injury are gathering considerable favour.

5.3.1 Evaluation of NT-proBNP in the anthracycline and non-anthracycline cohorts during chemotherapy

In an assessment of NT-proBNP measures in the early, late and continuous-dose anthracycline groups prior to commencement of chemotherapeutic regimen, no significant difference was identified ($p>0.05$; Kruskal-Wallis H test). Such findings were also demonstrated between all three anthracycline groups when evaluated at mid-treatment assessment or final measurement ($p>0.05$; Kruskal-Wallis H test).

In a specific assessment of NT-proBNP levels in each of the anthracycline groups, a significant increase was noted in the late-dose anthracycline group during chemotherapy ($**p<0.01$; Friedman test) which was in contrast to the comparable measures of the early-dose ($p>0.05$; Friedman test) and continuous-dose groups ($p>0.05$; Friedman test). However, the number of patients in the late-dose group was small (number of patients, $n=8$), with only five out of a total of 23 samples above the URL of 125 pg/mL.

Given the comparable measures observed across all three anthracycline groups, NT-proBNP levels in all three anthracycline groups were assessed collectively as the anthracycline cohort and were determined to be significantly different during the course of chemotherapy ($*p<0.03$; Friedman test). There was no significant difference in NT-proBNP measures in the non-anthracycline cohort during the course of chemotherapy ($p>0.05$; Friedman test).

In a comparison of the anthracycline cohort with the non-anthracycline cohort at all three time-points (naïve to treatment, mid-treatment and final measurement) a

significant difference in levels was demonstrated (** $p < 0.01$; Kruskal-Wallis H test; Figure 5.1). Post-hoc analysis using Dunn's multiple comparison test indicated a significantly lower NT-proBNP concentration in the anthracycline cohort mid-treatment when compared with the non-anthracycline cohort at naïve to treatment (** $p < 0.01$) and mid-treatment assessments (* $p < 0.05$).

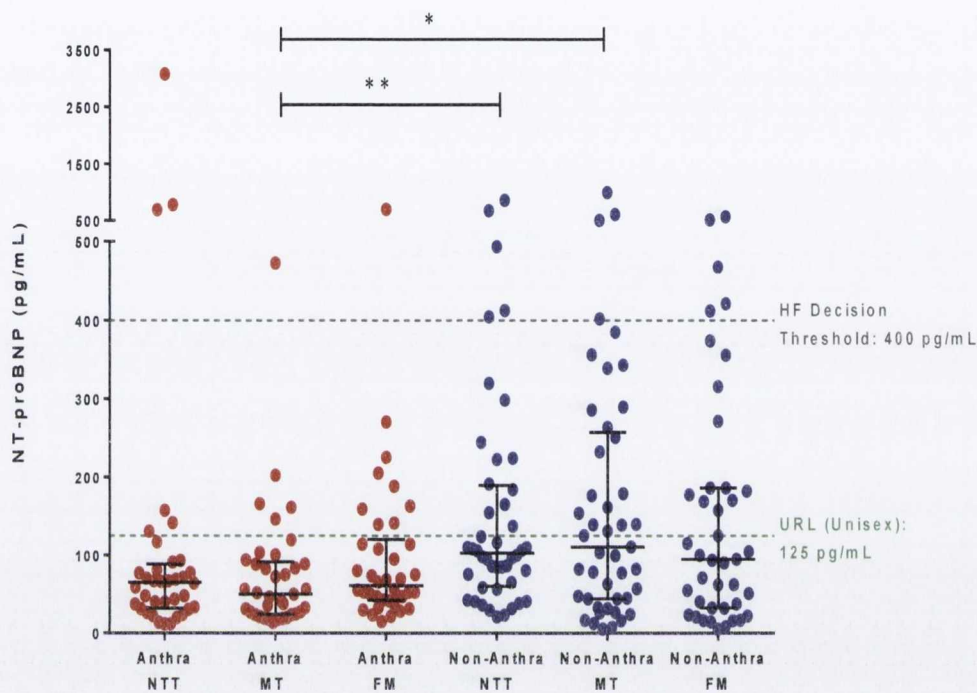


Figure 5.1 Evaluation of NT-proBNP levels in the anthracycline and non-anthracycline cohorts. NT-proBNP measures were significantly lower in the anthracycline cohort at mid-treatment assessment when compared the non-anthracycline cohort at naïve to treatment and mid-treatment assessments. Total number of patients, n=84; total number of samples, n=241. **p<0.01; Kruskal-Wallis H test, post-hoc analysis using Dunn's multiple comparison test. Results were expressed as median $\pm$ IQR. Anthra, anthracycline cohort; FM, final measurement; HF, heart failure; MT, mid-treatment; Non-Anthra, non-anthracycline cohort; NTT, naïve to treatment; URL, upper reference limit.

5.3.2 Specific assessment of elevated NT-proBNP measures in both study cohorts during chemotherapy

In the anthracycline cohort, the majority of measurements were significantly below the 125 pg/mL URL. Ten patients presented with values above this level; two had previously documented hypertension, one patient had two previous myocardial infarctions and one patient presented with arrhythmia. One patient presented with atrial fibrillation post-chemotherapy treatment. Three patients presented with an NT-proBNP above the 400 pg/mL decision threshold which would be considered indicative of heart failure. Of these patients, one patient had two previous myocardial infarctions and documented cardiac disease, while two patients had hypertension.

A total of 24 patients in the non-anthracycline cohort presented with NT-proBNP values above the 125 pg/mL URL, of which seven patients had hypertension and two others had ischemic heart disease. In an assessment of the NT-proBNP measures which were above the 400 pg/mL decision threshold for heart failure (number of samples, $n=10$), two patients had ischemic heart disease and one patient had hypertension, while two patients demonstrated values which were just above the 400 pg/mL decision threshold. No significant cardiac injury was documented in the remaining five patients.

5.3.3 Assessment of NT-proBNP measures based on renal function

Recent studies have highlighted an association between NT-proBNP measures and renal function, as assessed by eGFR. In this study, NT-proBNP measurements were correlated with eGFR calculated using CKD-EPI SCr. In the anthracycline cohort (number of patients, $n=38$), there was no significant correlation between NT-proBNP levels and eGFR ($r = -0.2520$; $p > 0.05$; 95% CI -0.4233 to -0.0633 ; Spearman's rank order correlation). However, in an assessment of NT-proBNP at eGFR measures below $60 \text{ ml/min/1.73m}^2$ which can be indicative of acute or chronic kidney disease, all three measures below this level were above the URL for NT-proBNP of 125 pg/mL (166, 226 and 3,080 pg/mL).

In a correlation of NT-proBNP measures with eGFR as calculated by CKD-EPI SCr in the non-anthracycline cohort (number of patients, n=46), a mild correlation was demonstrated ($r = -0.5403$; **** $p < 0.0001$; 95% CI -0.6548 to -0.4015 ; Spearman's rank order correlation). When NT-proBNP measures were evaluated at eGFR levels below $60 \text{ ml/min/1.73m}^2$, 14 out of 15 NT-proBNP values were above 125 pg/mL (median, 339 pg/mL ; IQR, 177 to 468 pg/mL ; range, 37.50 to 994.4 pg/mL).

5.3.4 Correlation between NT-proBNP and hs-cTnI concentration

It has been firmly established that both NT-proBNP and hs-cTnI are biomarkers of cardiac damage. In this evaluation, NT-proBNP a biomarker of cardiac strain was correlated with hs-cTnI, a biomarker of cardiac injury to detect evidence of an association between the biomarkers in cases of chemotherapy-mediated cardiotoxicity, as has been demonstrated previously between NT-proBNP and hs-cTnT (Kusumoto *et al.*, 2012). In an assessment of NT-proBNP and hs-cTnI measurements in the anthracycline cohort (total number of patients, n=38; total number of paired measurements, n=111), no distinct correlation between both biomarkers was determined ($r = 0.1876$; * $p < 0.05$; 95% CI -0.0043 to 0.3662 ; Spearman's rank order correlation).

A comparison of NT-proBNP measures when hs-cTnI in the non-anthracycline cohort demonstrated no distinguishable correlation between NT-proBNP and hs-cTnI (total number of patients, n=46; total number of paired measurements, n=129) ($r = 0.2961$; *** $p < 0.001$; 95% CI 0.1249 to 0.4503 ; Spearman's rank order correlation).

5.3.5 Determination of aldosterone concentration using LC-MS/MS

The LC-MS/MS assay developed in this study (see Chapter 3) to quantify aldosterone in patient samples is recognised to have a lower LOQ when compared with immunoassays used in current clinical practice. In this study, a total of 89 out of 219 (41%) measurements were found to be below the LOQ of the IDS-iSYS

chemiluminescent immunoassay (138 pmol/L), with 20 (9%) measurements found to be below the LOD of the LC-MS/MS immunoassay.

5.3.6 Evaluation of aldosterone measures in the anthracycline and non-anthracycline cohorts during chemotherapy

Cardiac injury mediated by chemotherapeutic agents including anthracyclines may be linked with aldosterone-mediated effects, as seen when decreases in LVEF arise following anthracycline treatment (Akpek *et al.*, 2015). In an assessment of aldosterone levels at initiation of chemotherapy in the anthracycline cohort, there was no significant difference between all three anthracycline groups ($p>0.05$; Kruskal-Wallis H test). Such findings were also noted when aldosterone was assessed at mid-treatment ($p>0.05$; Kruskal-Wallis H test) and final measurement ($p>0.05$; Kruskal-Wallis H test) intervals.

Aldosterone measurements demonstrated no significant variation during chemotherapy in the early-dose and late-dose anthracycline groups ($p>0.05$; Friedman test). There was a significant difference in aldosterone levels in the continuous-dose anthracycline group during the course of chemotherapy (** $p<0.01$; Friedman test), however there were only nine patients in this group, with final measurement levels found to be lower than baseline measures. In addition, all measures were below the URL of 1,314 pmol/L for aldosterone, defined using the Waters Xevo TQS LC-MS/MS method (assay procedure 2; see Chapter 3) in collaboration colleagues at Mater Laboratory. On account of the absence of demonstrable variation of the aldosterone levels in the anthracycline groups, all three groups were assessed collectively as the anthracycline cohort for comparison with the non-anthracycline cohort.

Aldosterone levels evaluated in the anthracycline and non-anthracycline cohorts separately demonstrated no significant difference during chemotherapy ($p>0.05$; Friedman test).

5.3.7 Comparison of aldosterone concentration in the oncology cohort during chemotherapy with a normal population

Aldosterone levels evaluated in the anthracycline and non-anthracycline cohorts were found to be comparable at all assessment intervals; baseline, mid-treatment and final measurement ($p>0.05$; Mann Whitney U test). Based on these findings, both groups were assessed collectively as the oncology cohort for comparison with a normal reference population.

When aldosterone levels in the oncology cohort were compared with measures from a normal population, there was a significant difference in aldosterone measurements between the groups (**** $p<0.0001$; Kruskal-Wallis H test; Figure 5.2). Post-hoc analysis using Dunn's multiple comparison test demonstrated aldosterone levels which were significantly lower in the oncology cohort at all assessment time-points when compared with a normal population (**** $p<0.0001$).

In an assessment of the three measures in the oncology cohort which were above the URL of 1,314 pmol/L, no significant cardiac injury was previously documented in these patients. An elevated NT-proBNP measure was identified in two of these patients, which indicates a possible link to cardiac injury.

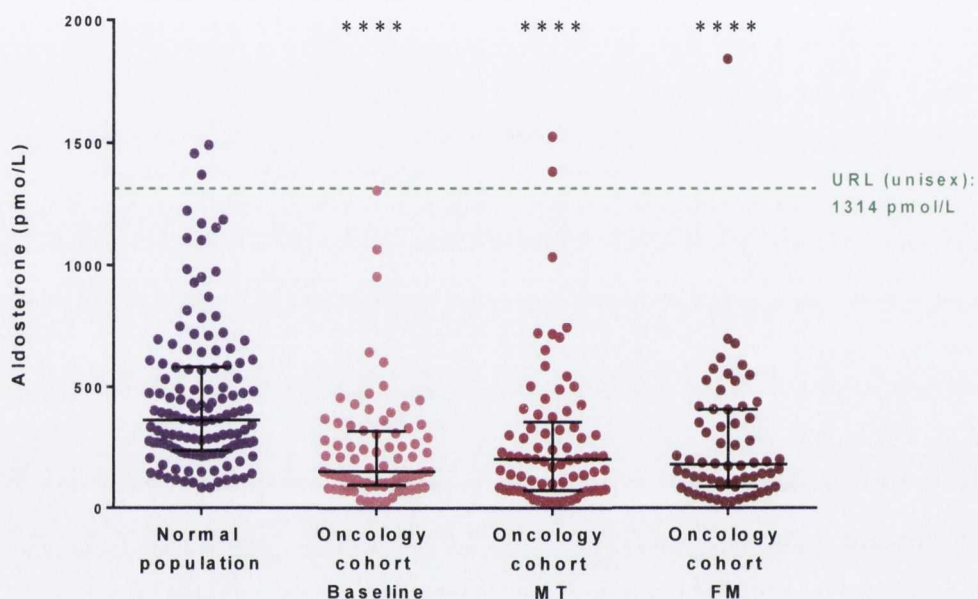


Figure 5.2 Comparison of aldosterone levels in the oncology cohort during chemotherapy with a normal population. Aldosterone measurements were significantly lower than the normal population at all assessment time-points in the oncology cohort, inclusive of anthracycline and non-anthracycline patients. Total number of patients, $n=214$; total number of samples, $n=353$. **** $p<0.0001$; Kruskal-Wallis H test, post-hoc analysis using Dunn's multiple comparison test. Results were expressed as median $\pm$ IQR. MT, Mid-treatment; FM, Final measurement.

5.3.8 Evaluation of galectin-3 measurements in the anthracycline groups

An assessment of galectin-3 levels prior to commencement of chemotherapy in the oncology cohort demonstrated no significant difference between all three anthracycline groups ($p>0.05$; Kruskal-Wallis H test). Galectin-3 measurements assessed during the middle of the chemotherapeutic regimen (mid-treatment) demonstrated a significant difference between all three anthracycline groups ($**p<0.01$; Kruskal-Wallis H test). Post-hoc analysis using Dunn's multiple comparison test indicated significant differences between the late-dose group and the other anthracycline groups ($**p<0.01$), with median values for each anthracycline group considerably less than the URL of 28 ng/mL (16 ng/mL (IQR, 10-20 ng/mL), 10 ng/mL (IQR, 9-12 ng/mL) and 18 ng/mL (16-22 ng/mL) for the early, late and continuous-dose anthracycline groups respectively). Comparable levels of galectin-3 were detected in all three anthracycline groups at final measurement ($p>0.05$; Kruskal-Wallis H test).

In a specific assessment of galectin-3 levels during chemotherapy in each of the anthracycline groups, no significant variation was observed in patients of the early-dose and continuous-dose anthracycline groups ($p>0.05$; Friedman test). While galectin-3 measurements were found to differ significantly during treatment in the late-dose anthracycline group ($*p<0.05$; Friedman test), post-hoc analysis using Dunn's multiple comparison test failed to detect a distinct difference between any of the assessment time-points. This was in addition to similar median values during assessment (baseline, 13.2 ng/mL (IQR, 11.2-18.4); mid-treatment, 9.7 ng/mL (IQR, 9.2-12.3); final measurement, 12.3 ng/mL (IQR, 8.4-16). While there were demonstrated differences in the late-dose anthracycline group, such differences were found to be negligible. On account of the relatively negligible differences observed in galectin-3 measures, all three anthracycline groups were assessed collectively as the anthracycline cohort for comparison with the non-anthracycline cohort.

5.3.9 Comparison of galectin-3 in the anthracycline and non-anthracycline cohorts during chemotherapy

The evaluation of galectin-3 in the anthracycline cohort demonstrated no significant variation in galectin-3 levels following chemotherapy ($p>0.05$; Friedman test), a finding which was also noted in the non-anthracycline cohort ($p>0.05$; Friedman test).

In a comparison of galectin-3 measures in both oncology cohorts during chemotherapy, significant differences were identified between the anthracycline and the non-anthracycline cohorts (**** $p<0.0001$; Kruskal-Wallis H test; Figure 5.3). Post-hoc analysis using Dunn's multiple comparison test demonstrated a significant difference in galectin-3 levels between the anthracycline cohort naïve to treatment and the non-anthracycline cohort at mid treatment assessment (* $p<0.05$), while significant differences were also noted between the anthracycline cohort at final measurement and the non-anthracycline cohort at all three assessment time-points (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Six patients from the anthracycline cohort presented with galectin-3 measures above the URL of 28 ng/mL. Two of these patients were found to also have an elevated NT-proBNP measure above 125 pg/mL. No patient was found to have an elevated aldosterone measurement above the URL. A total of nine patients were found to have an elevated galectin-3 measurement in the non-anthracycline cohort, with four of these patients also found to have an elevated NT-proBNP measures above 400 pg/mL, considered to be the clinical decision threshold for heart failure.

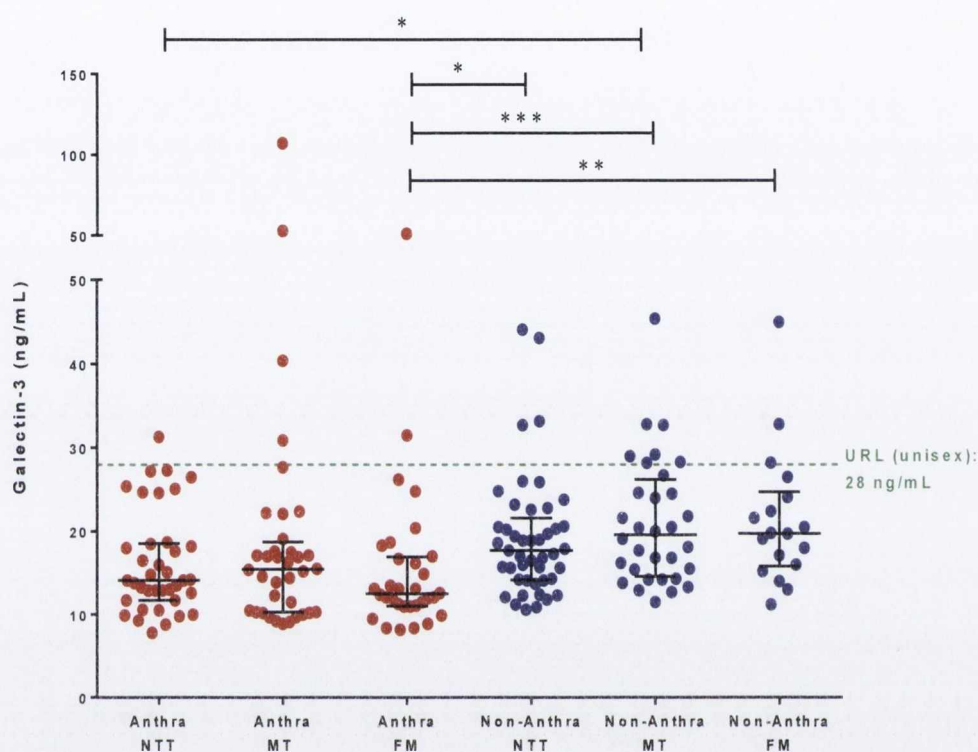


Figure 5.3 Evaluation of galectin-3 levels in the anthracycline and non-anthracycline cohorts during chemotherapy. Significant difference in galectin-3 measurements between anthracycline cohort at final measurement and the non-anthracycline cohort at all analytical time-points. Total number of patients $n=83$; total number of samples $n=201$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; Kruskal-Wallis H test, post hoc analysis using Dunn's multiple comparison test. Results were expressed as median $\pm$ IQR. Anthra, anthracycline cohort; FM, final measurement; HF, heart failure; MT, mid-treatment; Non-Anthra, non-anthracycline cohort; NTT, naïve to treatment; URL, upper reference limit.

5.3.10 Evaluation of galectin-3 levels in patients with elevated cardiac biomarkers and eGFR measures below 60 ml/min/1.73m²

Galectin-3 levels were assessed in patients where elevated cardiac biomarkers demonstrated indications of cardiac injury. In the anthracycline cohort, patients with an elevated hs-cTnI measurement above the gender-specific 99th percentile (female, 16 ng/L; male, 34 ng/L) were examined for associations with elevated galectin-3 levels. No significant correlation was identified between the peak hs-cTnI measurement for each patient and the peak galectin-3 measure ($r=0.1621$; $p>0.05$; 95% CI -0.28 to 0.5474; Spearman's rank order correlation) (total number of patients, $n=23$; total number of samples, $n=46$) or between the peak hs-cTnI for each patient and the final galectin-3 level ($r=0.002$; $p>0.05$; 95% CI -0.4209 to 0.4249; Spearman's rank order correlation) (total number of patients, $n=23$; total number of samples, $n=46$). No patient in the non-anthracycline cohort presented with an hs-cTnI measurement above the gender-specific 99th percentiles.

In an evaluation of NT-proBNP measurements above the URL of 125 pg/mL adopted for analysis of cardiac injury, no correlation was demonstrated in the anthracycline cohort between the peak NT-proBNP for each patient and the corresponding peak galectin-3 level ($r=0.2452$; $p>0.05$; 95% CI -0.3989 to 0.7273; Spearman's rank order correlation) (total number of patients, $n=12$; total number of samples, $n=24$). Additionally, there was no distinct correlation between the peak NT-proBNP level for each patient and final galectin-3 measurement ($r=0.2242$; $p>0.05$; 95% CI -0.4174 to 0.7166; Spearman's rank order correlation) (total number of patients, $n=12$; total number of samples, $n=24$).

In an evaluation of patients from the non-anthracycline cohort who demonstrated NT-proBNP levels above the 125 pg/mL URL, peak NT-proBNP and galectin-3 values for each patient were compared, demonstrating a moderate correlation between measurements ($r=0.5287$; $**p<0.01$; 95% CI 0.1469 to 0.7734; Spearman's rank order correlation) (total number of patients, $n=24$; total number of samples, $n=48$). In a correlation of the peak NT-proBNP measure for each patient and the final galectin-3 measurement, a moderate correlation was also

observed ($r=0.5649$; $**p<0.01$, 95% CI 0.1971 to 0.7933, Spearman's rank order correlation) (total number of patients, $n=24$; total number of samples, $n=48$).

While aldosterone measurements were also evaluated, only three values were above the URL of 1,314 pg/mL in the oncology cohort, which was not sufficient for a correlation with galectin-3. However, on assessment of elevated galectin-3 levels above the URL of 28 ng/mL documented in six patients of the anthracycline cohort, aldosterone measures were demonstrated to peak at the same measurement interval in five of the six patients. Only two of the 10 patients of the non-anthracycline cohort demonstrated peak galectin-3 levels above 28 ng/mL which corresponded with peak aldosterone measurements.

In an assessment of galectin-3 measurements prior to and during chemotherapy which presented above the URL (number of samples, $n=20$), only two measurements corresponded to an eGFR (CKD-EPI SCr) below 60 ml/min/1.73m².

5.3.11 Comparison of NT-proBNP, aldosterone and galectin-3 measures with ECHO

In an evaluation of the two patients in the anthracycline cohort diagnosed with Grade I diastolic dysfunction during the course of anthracycline chemotherapy, one patient demonstrated an NT-proBNP measure (163.3 pg/mL) above the URL of 125 pg/mL at final measurement. This patient also demonstrated a galectin-3 measurement (53 ng/mL) significantly greater than the URL of 28 ng/mL which coincided with the time of diagnosis. At completion of chemotherapy, the galectin-3 level remained significantly above the URL (51.3 ng/mL). In the case of the second patient from this cohort diagnosed with Grade I diastolic dysfunction, NT-proBNP and galectin-3 measures were considerably below the respective URL throughout chemotherapy (NT-proBNP range, 12 to 36 pg/mL; galectin-3 range, 10 to 13 ng/mL). An assessment of the patient from the non-anthracycline cohort diagnosed with Grade I diastolic dysfunction, there was no elevated NT-proBNP, aldosterone or galectin-3 measures above the URL.

In an evaluation of two other patients in the anthracycline cohort with suspected marginal decreases in LVEF, but failed to fulfil the criteria for cardiotoxicity as designated by the Cardiac Review and Evaluation Committee (CREC) (Seidman *et al.*, 2002; Fiuza, 2009), NT-proBNP and galectin-3 measures did not vary substantially with no values above the URL for either biomarker.

5.4 Discussion

Cardiotoxicity mediated by chemotherapy and targeted therapies can often cause significant levels of cardiac stress, often disrupting the harmony of cardiac myocyte regeneration in favour of more permanent cardiac myocyte damage, which can potentially induce a detrimental form of cardiac remodelling (Chen *et al.*, 2007). There is a growing recognition of the intricate pathophysiological mechanisms such as the neurohormonal and fibrotic processes which can result from and propagate these harmful effects. Recent studies have endorsed this hypothesis by virtue of the myriad of biomarkers which are now being investigated, each focusing on pathophysiological processes which can be affiliated with cardiac injury (Ky *et al.*, 2014; Putt *et al.*, 2015). NT-proBNP, aldosterone and galectin-3 are examples of such biomarkers which have been investigated for their sensitivity in detecting specific forms of cardiac injury.

This study demonstrated a marginally significant difference in NT-proBNP measures during chemotherapy treatment (* $p < 0.03$; Friedman test). While median NT-proBNP values remained comparable during chemotherapy treatment and were considerably below the URL of 125 pg/mL, the IQR range at final measurement was higher than at previous assessment time-points, but remained below the URL. This was in addition to comparable NT-proBNP measures observed in the early-dose and continuous-dose anthracycline groups. Where differences were noted in the late-dose group, patient numbers were too small to conclude on the significance of the result which was supplementary to the fact that this group received smaller median anthracycline doses when compared with early-dose and continuous-dose groups and therefore the findings from this cohort were inconclusive. The results from this study were similar to studies published where no definitive diagnostic or monitoring value for NT-proBNP assessment could be ascertained, by virtue of the comparable measures of NT-proBNP identified following treatment with doxorubicin and cyclophosphamide in 78 breast cancer patients (Ky *et al.*, 2014). A longer-term evaluation of NT-proBNP over a period of 15 months in this patient cohort also failed demonstrate any association between this biomarker and cardiotoxicity as defined by the Cardiac Review and Evaluation Criteria (Putt *et al.*, 2015). In contrast, Kittiwarawut and

colleagues demonstrated significant associations between NT-proBNP levels and decreases in LVEF indicative of cardiotoxicity using the National Cancer Institute common toxicity criteria version 2.0 (Kittiwara-wut *et al.*, 2013). However the number of patients in this study was small (number of patients, n=53) and only 11 patients demonstrated evidence of cardiac injury.

A significant difference was noted in NT-proBNP measurements between the anthracycline and non-anthracycline cohorts. Median and IQR values were always noted to be higher in the non-anthracycline cohort and may be in part attributable to the older age of the non-anthracycline cohort (***p<0.001; unpaired t-test). As NT-proBNP has previously been reported to increase with age (Bay *et al.*, 2003), this may account for the difference noted between both cohorts. The older age profile of patients in the non-anthracycline cohort may have explained the higher NT-proBNP values as kidney function deteriorates with age. While age-specific URLs for NT-proBNP have been utilised previously, the sample size in this study was not sufficient to allow for the thorough assessment of the influence of age-specific cut-offs in the detection of anthracycline-mediated cardiac injury. In cases where measures were found to be elevated above the URL or HF clinical decision threshold, there was a link with previous cardiac history.

A growing body of evidence has highlighted an association between elevated NT-proBNP levels and decreased renal function, with the presence of cardiovascular diseases further potentiating increases of NT-proBNP (deFilippi *et al.*, 2005). In this study, no distinct correlation was found between both entities, however there was a trend for NT-proBNP values to be above the 125 pg/mL URL when eGFR was below 60 ml/min/1.73m², demonstrated in 17 out of 18 measurements identified below this eGFR limit. While a correlation to assess this relationship would be desirable, the number of samples which correlated to an eGFR below 60 ml/min/1.73m² was small. As eGFR levels below 60 ml/min/1.73m² are strongly associated with acute and chronic kidney disease, the elevated levels of NT-proBNP observed in such cases likely reflect diminished clearance and cannot be attributable to direct effects of chemotherapy.

There was no demonstrable correlation between NT-proBNP and hs-cTnI in the anthracycline ($r=0.1876$; Spearman's rank order correlation) or non-anthracycline ($r=0.2961$; Spearman's rank order correlation) cohorts in this study. While correlations between cardiac troponin and NT-proBNP were previously demonstrated by the moderate correlation between the log-transformed values of hs-cTnT and NT-proBNP in heart failure patients (Kusumoto *et al.*, 2012), such findings were not noted in this study. Based on the findings from the study by Kusumoto, cardiac wall stretch and cardiac myocyte injury may coincide, resulting in a reasonable association between both biomarkers. A reasonable explanation for the absence of a correlation in this study is related to the mechanism of anthracycline-mediated cardiac injury detected by hs-cTnI in this study, which is that of cardio-myocyte injury. This theory is reinforced by the findings by Ky (Ky *et al.*, 2014) depicting elevated ultrasensitive cTnI and not NT-proBNP measures in patients receiving similar anthracycline-based chemotherapeutic regimen. When coupled with the potential influence of age and renal function, particularly at levels indicative of acute and chronic kidney disease, the findings of this study highlight the lack of benefit to correlating both biomarkers for the detection of cardiac injury mediated by anthracycline-based chemotherapy.

In an assessment of aldosterone in this study, the relatively stable measures determined in the oncology cohort during chemotherapy treatment indicate that subclinical cardiac injury as detected in the anthracycline cohort using hs-cTnI did not result in any significant influence on aldosterone levels, as measures were consistently comparable with the non-anthracycline cohort. Furthermore, when studied as a single oncology cohort, aldosterone levels were found to be significantly lower in this cohort when compared with a normal population. Garrone published a study with similar findings following assessment of cTnI and aldosterone levels in 50 patients who received a combination chemotherapeutic regimen containing epirubicin, thought to be a safer alternative to doxorubicin (Garrone *et al.*, 2011). This study using a novel sensitive assay for aldosterone corroborates findings by Garrone and colleagues and highlights the fact that

aldosterone does not provide an efficient means of detecting subclinical cardiac injury based on the experiences in this study.

The LC-MS/MS assay utilised in this study facilitated a more accurate and robust means of quantifying aldosterone compared with the new immunoassay with poorer analytical characteristics, including higher LOQs and potential interferences. In this study, over 40% of measurements were below the LOQ of the IDS-iSYS immunoassay utilised in current practice in the Mater Diagnostic laboratory. As such, these measurements would have been potentially significantly overestimated in this study by assigning the LOD of the IDS immunoassay to each value which could not be quantified. The LC-MS/MS assay facilitated a more robust means of assessment, through determining the concentration of aldosterone in a significantly greater number of samples, which thereby promotes this LC-MS/MS as the optimal method of choice for both oncology and other patient cohorts.

The clinical utility of aldosterone may have a role in the assessment of cardioprotective strategies for patients receiving anthracycline treatment. Many of these cardioprotective strategies significantly impact the renin-angiotensin-aldosterone system and in the case of agents such as spironolactone examined previously (Akpek *et al.*, 2015) will have a direct impact on aldosterone levels. By quantifying aldosterone measurements using an LC-MS/MS assay with an analysis time of less than five minutes, the efficacy of cardioprotective strategies by means of aldosterone assessment can be investigated in a timely manner.

Galectin-3 did not demonstrate indications of cardiac injury when assessed during chemotherapy in patients of the anthracycline or non-anthracycline cohorts. There was no significant changes to measurements during chemotherapy in either cohort ($p > 0.05$; Friedman test) which were epitomised by similar median and IQR values during chemotherapy, which demonstrated that cardiac injury detected by increases in hs-cTnI in the anthracycline cohort had not resulted in increases in galectin-3. This finding was expected as chemotherapy is unlikely to induce any demonstrable levels of cardiac fibrosis early in the course of treatment. The results in this study have corroborated findings from Ky and colleagues which

failed to demonstrate any significant changes in galectin-3 levels in breast cancer patients receiving an anthracycline-based chemotherapeutic regimen (Ky *et al.*, 2014). When galectin-3 levels were assessed relative to the baseline measurement up to 15 months post-commencement of chemotherapy, no significant difference was determined (Putt *et al.*, 2015). Although the change in galectin-3 measures between assessment intervals demonstrated an association with cardiotoxicity, the result was of marginal significance and would warrant evaluation in a larger study cohort. Each anthracycline group demonstrated relatively comparable measures during treatment. The late-dose group appeared to demonstrate a statistically significant difference to the other anthracycline groups when galectin-3 was assessed mid-way through the chemotherapeutic regimen (** $p < 0.01$; Kruskal-Wallis H test). However, this difference was minimal with similar median values observed during chemotherapy with post-hoc analysis failing to indicate any particularly significant difference between groups. As such, galectin-3 measures were deemed to be relatively stable during treatment, with no significant indications of cardiac injury.

There was no significant relationship between elevated hs-cTnI and galectin-3 measurements in the anthracycline and non-anthracycline cohorts, indicating the absence of a direct relationship between both biomarkers. These findings suggest that the anthracycline-based chemotherapeutic regimen did not induce a fibrotic process in the heart or in any other organ during the early course of chemotherapy. NT-proBNP has previously been shown to be associated with cardiotoxicity, particularly left ventricular damage (Sandri *et al.*, 2005) with increased levels previously reported in patients receiving anthracycline chemotherapy (Kittiwarawut *et al.*, 2013) which correlated with decreases in LVEF. In this study, NT-proBNP, aldosterone and galectin-3 were investigated in patients who presented with decreases in LVEF during chemotherapy, there was no demonstrable association between echocardiographic findings and biomarker levels. This study is limited by the small number of patients with clinical signs associated with deterioration in cardiac function and knowledge as to their blood pressure at the time of aldosterone assessment. A plausible reason for the lower aldosterone levels in the oncology cohort was that almost all of the patients were

post-menopausal women (or men) and therefore were not affected by cyclical effects of progesterone in the luteal phase, known to be associated with higher aldosterone levels.

The conclusion from this study is that the role for NT-proBNP, aldosterone and galectin 3 in patients receiving chemotherapy is reserved for those with pre-existing HF, onset of clinical signs of HF and, or hypertension during treatment, however, these biomarkers may have a useful role in monitoring patients receiving cardio-protective therapies.

Chapter 6

Evaluation of novel renal biomarker applications in patients receiving chemotherapy

6.1. Introduction

Patients receiving chemotherapeutic regimen are recognised to be at substantial risk of impaired renal function during the course of chemotherapy (Launay-Vacher *et al.*, 2007) which warrants the use of robust diagnostic methods to facilitate detection. The serial assessment of cystatin C and NGAL provide a new means to evaluate renal function and detect acute kidney injury respectively. Additionally, the recently endorsed CKD-EPI formulae used to estimate GFR demonstrates considerable promise in monitoring renal function, detecting kidney injury and chemotherapeutic drug dosing.

Cystatin C has been shown to demonstrate superior potential to creatinine in swiftly indicating renal impairment (Christensson *et al.*, 2003), with levels independent of dietary protein intake (Tangri *et al.*, 2011) and muscle mass (Vinge *et al.*, 1999), which may be critical as oncology patients may experience poor appetite and significant changes in muscle mass during treatment.

The CKD-EPI equations have recently been promoted for the routine estimation of GFR (Levey *et al.*, 2009; Inker *et al.*, 2012). These formulae have demonstrated the closest estimation to the gold-standard of mGFR in an oncology cohort when compared with other renal function estimating formulae, whilst also indicating significant potential in improving chemotherapeutic drug dosing (Chew-Harris *et al.*, 2015). Further improvements in dosing concordance are proposed with the removal of BSA indexing, in cases where drug doses are calculated according to body size or weight (Levey *et al.*, 2015). This recommendation was recently supported by observations of superior dosing concordance for carboplatin using the Calvert formula, when BSA indexing was removed from CKD-EPI and compared with mGFR-calculated doses (Dooley *et al.*, 2013).

NGAL has demonstrated considerable potential as a renal injury biomarker, specifically in the detection AKI (Nickolas *et al.*, 2008; Soto *et al.*, 2013). The utility of NGAL in oncology populations, known to have generally low neutrophil counts, may be emphasised in these patients in the event of renal impairment,

where NGAL levels would be strongly suspected to correlate with release from the renal tubules and not from neutrophils.

In this study, cystatin C was evaluated for the assessment of renal function with comparison to creatinine, while NGAL was assessed for the detection of AKI. The newly promoted CKD-EPI formulae were also compared with the currently utilised CG and MDRD formulae, including in the calculation of carboplatin drug doses using the Calvert formula, with and without the addition of BSA indexing.

The aims of this study were:

- To determine cystatin C measurements in an oncology cohort prior to and during chemotherapy treatment and to compare to creatinine measurements
- To evaluate the application of creatinine and cystatin C in CG-CrCl and eGFR equations and to assess formula performance in an oncology cohort
- To investigate the use of CG-CrCl and CKD-EPI SCr in the dosing of chemotherapeutic agents using the Calvert formula
- To identify the utility of NGAL to detect kidney injury in the oncology cohort during chemotherapy

6.2. Methods

A serum and urine sample was collected from all 84 patients prior to commencement of each cycle of chemotherapy treatment. Samples collected were centrifuged at 3,500 g for 10 minutes at room temperature and the serum was aliquoted and frozen at -70°C until analysis.

Serum aliquots taken from each treatment cycle were defrosted, mixed and centrifuged at 3,500 g for 10 minutes before being analysed using a particle-enhanced turbidimetric immunoassay (PETIA) for cystatin C (n=496) (Abbott Architect c16000) and using an enzymatic assay for creatinine (n=524) (Abbott Architect c16000) (see section 2.1.2.1). Urine samples were defrosted, mixed and centrifuged before analysis using a 2-step immunoassay for NGAL (Abbott Architect i1000sr) (see section 2.1.2.1).

Carboplatin doses, patient weight and BSA details were obtained directly from pharmacy records. CrCl as estimated by Cockcroft-Gault and eGFR as determined by MDRD and CKD-EPI equations (SCr, serum creatinine; CysC, cystatin C; SCr/CysC, serum creatinine/cystatin C combined) were calculated for each patient naïve to treatment and up to the eighth cycle of chemotherapy.

Results were tested for normality using the D'Agostino-Pearson omnibus normality test. Grubb's test was used to detect significant outlier data. Parametric statistical tests were applied in the assessment of biomarkers from the normal population; unpaired t-test. Results were presented as mean $\pm$ SD. Non-parametric statistical tests were applied in the assessment of biomarkers from the oncology cohort; Wilcoxon signed-rank test (paired t-test), Mann-Whitney U test (unpaired t-test), Kruskal-Wallis H test (one-way ANOVA) and Friedman test (repeated measures ANOVA). Results were presented as median $\pm$ IQR. Post-hoc analysis was performed using Dunn's multiple comparison test. Bland-Altman tests were used to assess the level of agreement between eGFR and CG-CrCl methods in the assessment of renal function and also specifically between CG and CKD-EPI SCr in the calculation of carboplatin drug doses with and without the addition of BSA indexing in the CKD-EPI SCr formula.

6.3. Results

In oncology, while creatinine is widely utilised in GFR estimating formulae (Perrone *et al.*, 1992), a drawback to its use is the influence of non-renal factors which includes changes in muscle mass (Baxmann *et al.*, 2008), which may mediate decreases in creatinine concentration. The uncertainty in creatinine measurement has prompted the investigation of cystatin C, suggested to be less susceptible to such interferences. The adoption of multiple GFR estimating formulae in oncology, MDRD in laboratory and CG in pharmacy assessment, has also promoted inaccuracy and complexity in assessing renal function, with considerable implications for chemotherapeutic drug dosing. CKD-EPI demonstrates improved precision and accuracy when compared with both formulae (Levey *et al.*, 2009; Michels *et al.*, 2010) and has the potential to more accurately detect renal impairment and adjust drug doses accordingly. The aim of these experiments was to evaluate the utility of cystatin C in an oncology cohort compared with creatinine, to evaluate NGAL in the detection of AKI, to assess MDRD and CG in current practice with the proposed CKD-EPI formulae and to evaluate its influence in carboplatin dosing.

6.3.1 Evaluation of creatinine and cystatin C in a normal population

In order to evaluate levels of cystatin C and creatinine in an oncology cohort, levels were firstly assessed in a normal population. Creatinine concentrations were found to be significantly lower in females (number of females, n=88) when compared with males (number of males, n=110) (****p<0.0001; unpaired t-test; Figure 6.1.A). Similarly, cystatin C also demonstrated significantly lower levels in females (number of females, n=119) when compared with males (number of males, n=120) (****p<0.0001; unpaired t-test; Figure 6.1.B).

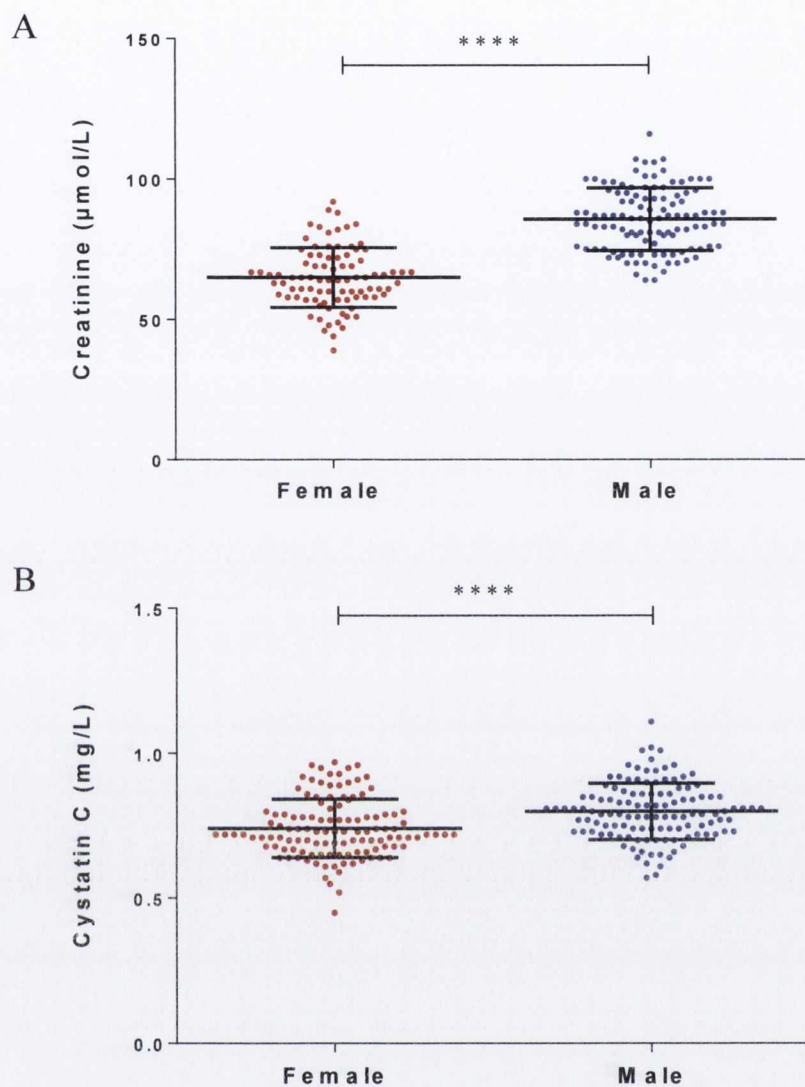


Figure 6.1 Gender-related differences for creatinine (A) and cystatin C (B) in a normal population. Concentrations of creatinine and cystatin C were significantly higher in males when compared with females. Total number of patients, n=198 (A) and 239 (B); total number of samples, n=198 (A) and 239 (B). ****p<0.0001; unpaired t-test. Results were expressed as mean $\pm$ SD.

6.3.2 Evaluation of creatinine and cystatin C in an oncology cohort

While cystatin C has been proposed as a suitable alternative to creatinine, recent publications have demonstrated elevated measures in malignancy including in myeloma, breast and prostate cancer (Terpos *et al.*, 2009; Tumminello *et al.*, 2009), which is suggestive of a malignancy-specific elevation in levels. In light of these findings, the suitability of cystatin C as a biomarker of renal function in oncology was examined in a cohort of oncology patients, prior to and during chemotherapy treatment. Creatinine levels were examined for comparison. Baseline cohort characteristics are presented in Table 6.1.

Table 6.1 Assessment of baseline study cohort characteristics. Results were expressed as median values, with the exception of patient age and BSA.

Patient characteristics	Total study cohort	Female	Male
Patient number	84	66	18
Age (years)	55	53	62
Actual body weight (kg)	70.55	66.65	81.45
BSA (m ²)	1.76	1.71	1.92
Baseline biomarker characteristics			
Serum creatinine (μmol/L)	63	63	84
Serum cystatin C (mg/L)	0.84	0.83	0.88
Baseline eGFR (ml/min/1.73m ²)			
CG (ml/min)	108	109	100
MDRD	88	89	81
CKD-EPI SCr	97	98	86
CKD-EPI CysC	94	96	90
CKD-EPI SCr/CysC	97	98	87

Creatinine levels measured in female oncology patients (number of patients, n=63) revealed comparable concentrations to a normal female population prior to commencing chemotherapy and following the first five cycles of treatment ($p>0.05$; Kruskal-Wallis H test; Figure 6.2.A). With additional assessment of this data, no significant changes during chemotherapy treatment were identified ($p>0.05$; Friedman test).

Cystatin C measurements in female oncology patients (number of patients, n=59) were significantly elevated prior to and following the first five cycles of chemotherapy when compared with a normal female population (**** $p<0.0001$; Kruskal-Wallis H test; Figure 6.2.B). Post-hoc analysis revealed a significant difference at each chemotherapeutic cycle when compared with normal females (* $p<0.05$; ** $p<0.01$; **** $p<0.0001$). Additional assessment of this data also revealed a significant increase in cystatin C concentration following the fifth cycle of chemotherapy when compared with the first cycle (* $p<0.0180$; Friedman test), thus chemotherapy treatment was found to be influencing cystatin C levels.

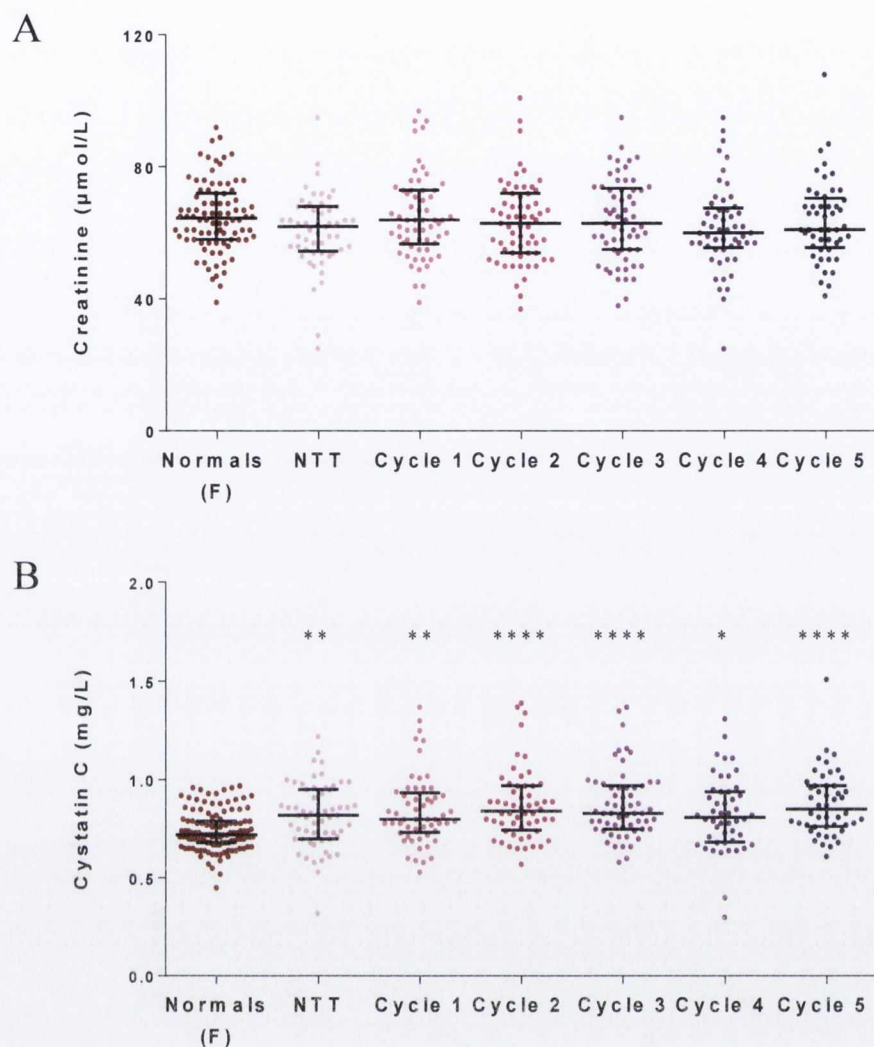


Figure 6.2 Creatinine (A) and cystatin C (B) concentrations in female oncology patients during the first five cycles of chemotherapy compared with a normal female population. Comparable concentrations of creatinine contrasted with significantly elevated levels of cystatin C during chemotherapy in female oncology patients. Total number of patients, $n=151$ (A) and 178 (B); total number of samples, $n=429$ (A) and 445 (B). * $p<0.05$; ** $p<0.01$; **** $p<0.0001$; Kruskal-Wallis H test, post-hoc analysis using Dunn's multiple comparison test. Results were expressed as median $\pm$ IQR. F, females; NTT, naïve to treatment.

Creatinine levels measured in male oncology patients (number of patients, n=15) demonstrated comparable concentrations to a normal female population prior to and following chemotherapy ($p>0.05$; Kruskal-Wallis H test; Figure 6.3.A). No significant changes in creatinine during chemotherapy treatment were noted ($p>0.05$; Mann-Whitney U test).

Cystatin C levels evaluated in male oncology patients (number of patients, n=16) prior to and during chemotherapy revealed significantly higher concentrations when compared with a normal male population (**** $p<0.0001$; Kruskal-Wallis H test; Figure 6.3.B). Post-hoc analysis identified significant differences between male oncology patients and a normal male population both prior to treatment (** $p<0.01$) and during chemotherapy (**** $p<0.0001$). No significant changes in cystatin C concentration were noted prior to or during chemotherapy treatment.

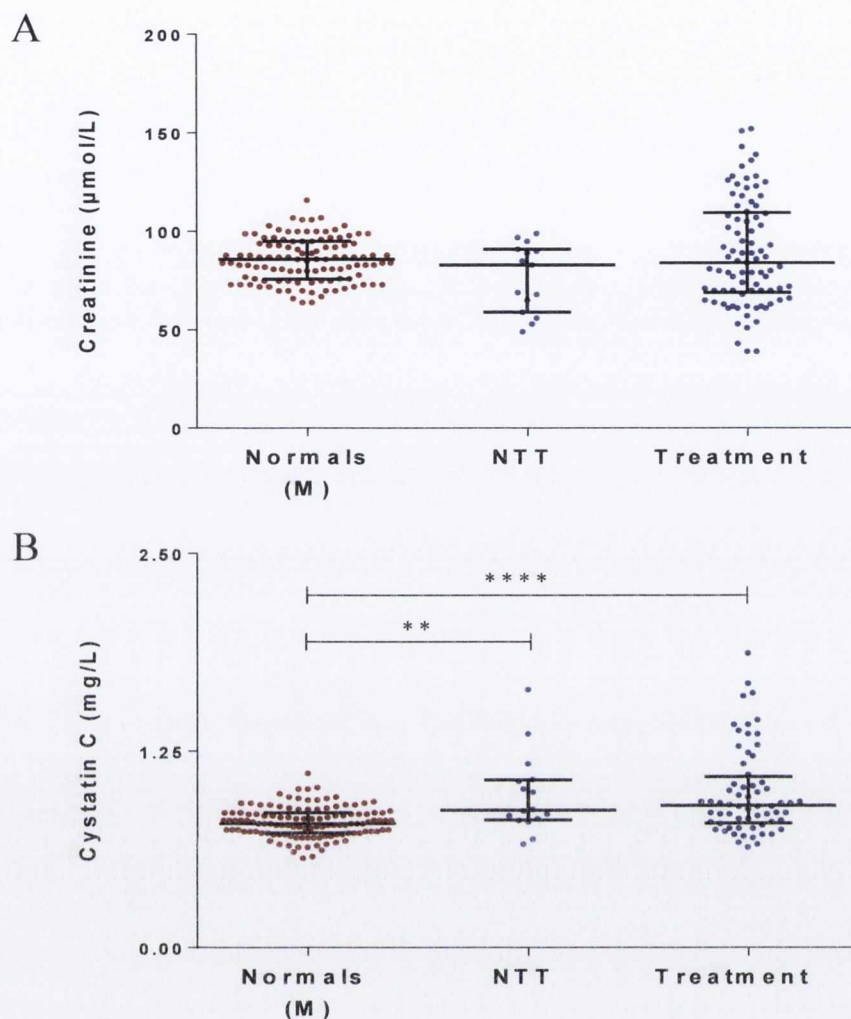


Figure 6.3 Creatinine (A) and cystatin C (B) concentrations in male oncology patients naïve to treatment and during chemotherapy compared with a normal male population. Comparable measures of creatinine contrasted with significantly elevated cystatin C levels during the course of chemotherapy in male oncology patients. Total number of patients, $n=125$ (A) and 136 (B); total number of samples, $n=210$ (A) and 200 (B). ** $p<0.01$; **** $p<0.0001$; Kruskal-Wallis H-test, post-hoc analysis using Dunn's multiple comparison test. Results were expressed as median $\pm$ IQR. M, males; NTT, naïve to treatment.

6.3.3 Assessment of cystatin C in different malignancies

To further elucidate the relationship between malignancy and elevated cystatin C concentrations, measurements were compared by cancer diagnosis with a normal population. For comparative purposes, creatinine was assessed in unison.

An assessment of creatinine measurements in female oncology patients grouped by malignancy and a normal female population identified significant differences between the groups ($***p<0.0005$; Kruskal-Wallis H test; Figure 6.4.A). Post-hoc analysis using Dunn's multiple comparison test demonstrated higher creatinine measures in ovarian cancer patients in comparison to a normal female population and breast cancer patients, however the small number of ovarian cancer patients could be mediating such differences ($*p<0.05$; $***p<0.001$).

A significant difference in cystatin C measurements was also found when female oncology patients were grouped by malignancy compared with a normal female population ($****p<0.0001$; Kruskal-Wallis H test; Figure 6.4.B). Post-hoc analysis demonstrated significantly higher concentrations of cystatin C in breast and lung cancer patients when compared with a normal female population ($****p<0.0001$), with a significant difference between lung and ovarian cancer also noted ($*p<0.05$).

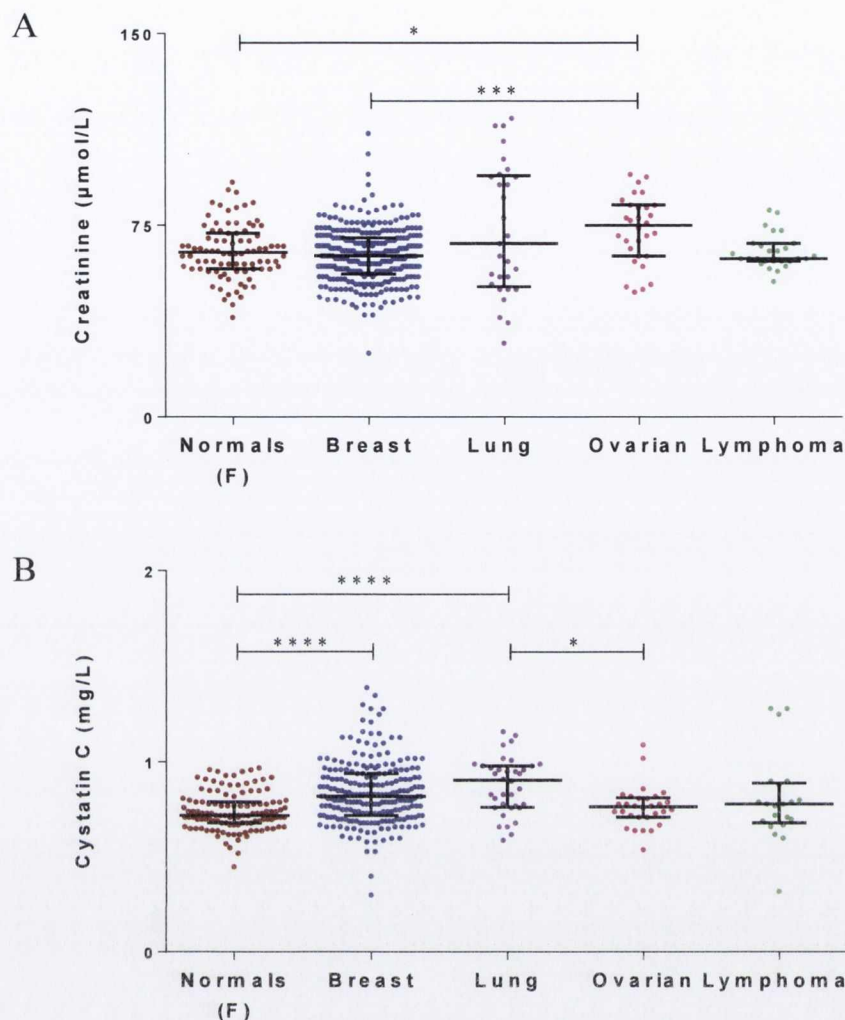


Figure 6.4 Evaluation of creatinine (A) and cystatin C (B) measures in female oncology patients according to malignancy when compared with a normal female population. Cystatin C measurements were significantly elevated in female breast and lung cancer patients when compared with a normal female population. Significant differences in creatinine and cystatin C were also noted for ovarian cancer, although sample numbers were small. Total number of patients, $n=148$ (A) and 179 (B); total number of samples, $n=477$ (A) and 432 (B). * $p<0.05$; *** $p<0.001$; **** $p<0.0001$; Kruskal-Wallis H test, post-hoc analysis using Dunn's multiple comparison test. Results were expressed as median $\pm$ IQR. F, females.

Cystatin C concentrations were evaluated in breast cancer patients (number of patients, n=45) during the course of chemotherapy. A significant difference was revealed following the first five cycles of treatment (**p<0.01; Friedman test; Figure 6.5). Post-hoc analysis indicated a significant increase in cystatin C following the fifth cycle of chemotherapy when compared with the first cycle (*p<0.05).

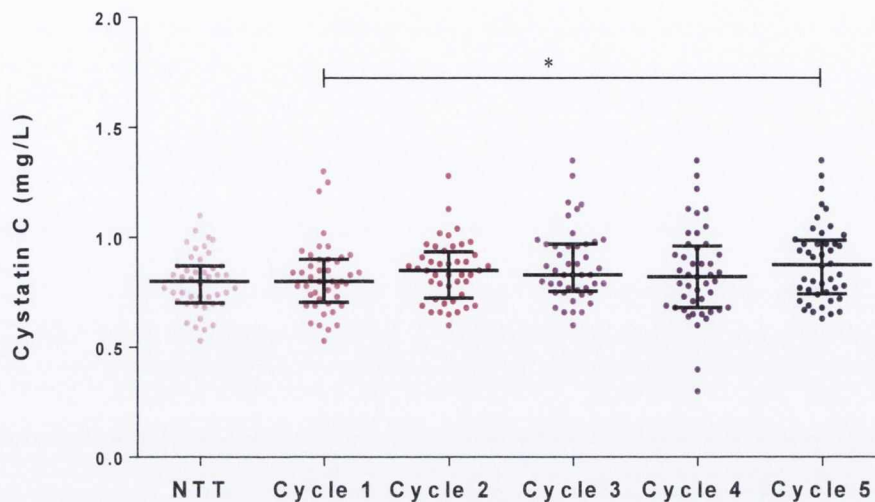


Figure 6.5 Cystatin C concentrations in female breast cancer patients prior to commencing chemotherapy and following the first five cycles of treatment. Cystatin C concentrations in the female breast cancer group increased significantly between the first and fifth cycles. Total number of patients $n=45$; total number of samples $n=266$. $*p<0.05$; Friedman test, post-hoc analysis using Dunn's multiple comparison test. Results were expressed as median $\pm$ IQR. NTT, naïve to treatment.

6.3.4 Assessment of CG-CrCl and eGFR formulae performance

For patients receiving chemotherapy, assessment of renal function is a critical aspect of therapeutic monitoring with estimations of CG-CrCl and GFR, namely MDRD among the most commonly utilised methods to detect signs of renal impairment and to calculate drug doses accordingly. However, despite recommendations in Kidney Disease: Improving Global Outcomes (KDIGO) guideline promoting the use of CKD-EPI (Global & Group., 2013), CG and MDRD remain in clinical use, despite their inaccuracy and lack of precision across the analytical range. To evaluate current practice in oncology, the currently utilised methods of CG and MDRD were compared with CKD-EPI to evaluate formula performance and reveal the discrepancies between methods, which may have implications for chemotherapeutic drug dosing.

An assessment of the performance of CG, MDRD and CKD-EPI equations (CKD-EPI SCr, CKD-EPI CysC and CKD-EPI SCr/CysC) among all oncology patients prior to and during chemotherapy revealed a significant difference in the assessment of renal function (**** $p < 0.0001$; Kruskal-Wallis H test; Figure 6.6). Post-hoc analysis using Dunn's multiple comparison test demonstrated significant differences when CG was compared with MDRD and CKD-EPI equations (** $p < 0.001$; **** $p < 0.0001$). MDRD was also significantly different to CKD-EPI SCr ($\delta\delta p < 0.01$) and CKD-EPI SCr/CysC ($\delta p < 0.05$).

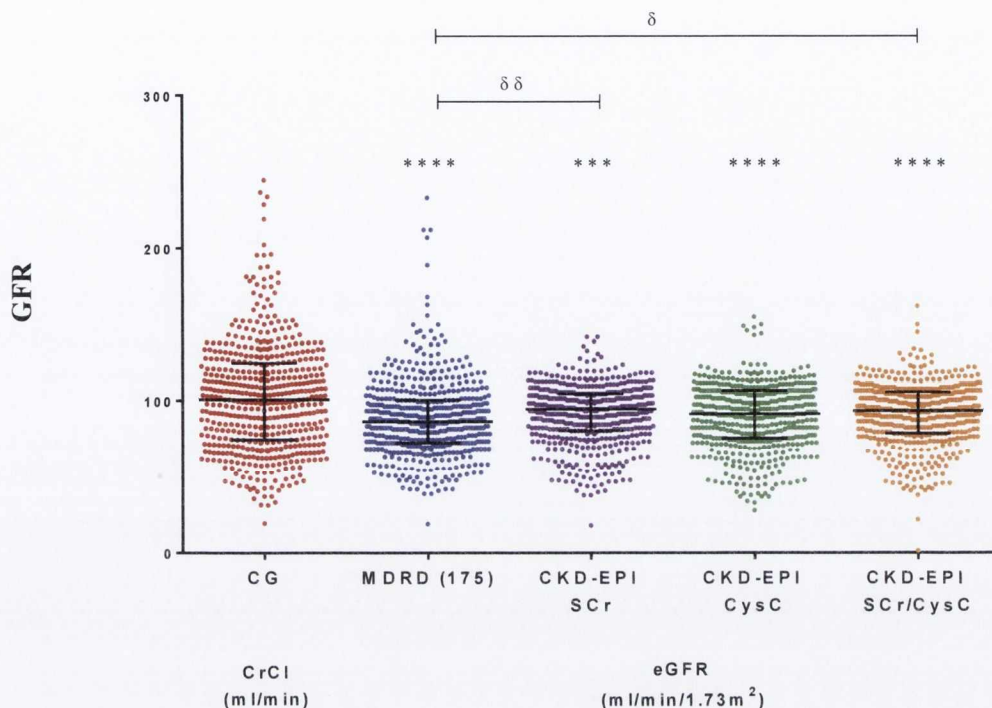


Figure 6.6 Comparison of CG-CrCl and eGFR formulae as measured by MDRD and CKD-EPI equations in an oncology cohort prior to and during chemotherapy. CG demonstrated significant differences with all eGFR formulae, while MDRD was also significantly discordant with SCr-based CKD-EPI formulae. Total number of patients, $n=84$; total number of samples, $n=2524$. Significant differences between CG and all other formulae were represented by (*), ($***p<0.001$; $****p<0.0001$), with significant differences between MDRD and CKD-EPI formulae represented by (δ) ($\delta p<0.05$; $\delta\delta p<0.01$); Kruskal-Wallis H test, post-hoc analysis using Dunn's multiple comparison test. Measurements were expressed as median $\pm$ IQR.

6.3.5 Comparison of current laboratory practice with pharmacy protocol and a comparison with the newly recommended CKD-EPI SCr

The GFR estimating formulae commonly used in the evaluation of oncology patients, which includes MDRD reported by most laboratories and CG-CrCl by pharmacy departments have been acknowledged to demonstrate considerable variation in assessing renal function. To evaluate the discordance between methods, MDRD measurements below $90 \text{ ml/min/1.73m}^2$ were compared by Bland-Altman plots with CG and CKD-EPI SCr.

The level of agreement between MDRD ($<90 \text{ ml/min/1.73m}^2$) and CG revealed 95% limits of agreement from -57.90 to 31.31 with a 13% negative bias (SD, 22.76) (Figure 6.7.A). Similarly, comparison of MDRD ($<90 \text{ ml/min/1.73m}^2$) and CKD-EPI SCr revealed 95% limits of agreement from -16.08 to 1.81 with a negative bias of 7% (SD, 4.56) (Figure 6.7.B).

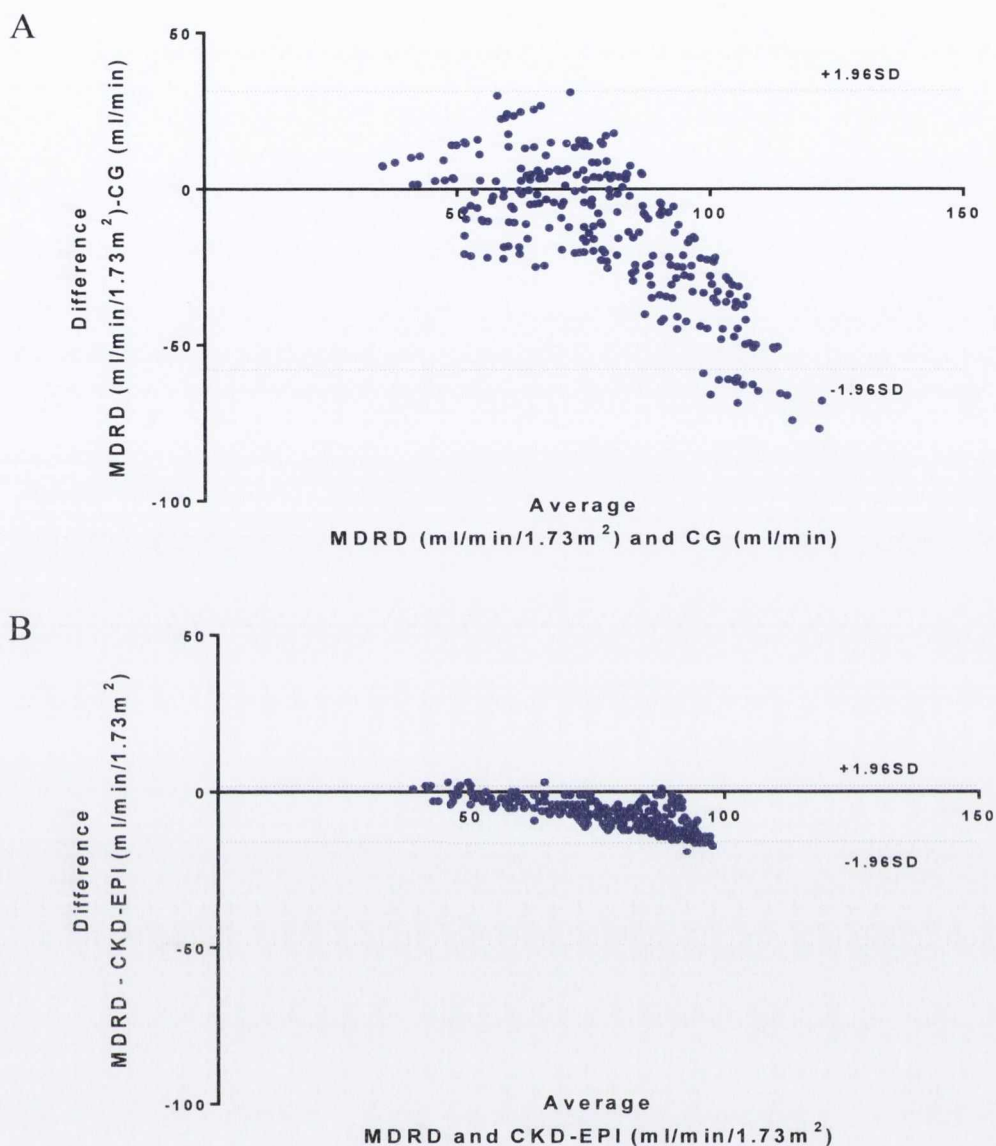


Figure 6.7 Bland-Altman plots of MDRD (<90 ml/min/1.73m²) with CG (A) and CKD-EPI SCr (B). At eGFR values calculated by MDRD below 90 ml/min/1.73m², there is much less discordance between MDRD and CKD-EPI than observed between MDRD and CG. Total number of patients, n=67 (A) and 67 (B); total number of samples, n=290 (A) and 290 (B). The dotted lines indicate the 95% limits of agreement for both methods (-57.90 to 31.31 (A) and -16.08 to 1.81 (B)).

6.3.6 Evaluation of differences in MDRD assessment

While MDRD has been widely adopted by laboratories, Bland-Altman plots have demonstrated significant differences when compared with CG and CKD-EPI (see Figure 6.7). Recent evidence demonstrates variability in the performance of this formula across the eGFR range (Matsushita *et al.*, 2012). In order to examine such differences, eGFR measurements below 60 ml/min/1.73m², between 60-90 ml/min/1.73m² and between 90-120 ml/min/1.73m² as calculated by MDRD were compared with CG and CKD-EPI.

eGFR measurements below 60 ml/min/1.73m² were found to be comparable with measurements calculated by CG or CKD-EPI ($p > 0.05$; Kruskal-Wallis H test; Figure 6.8). However, whilst no significant difference was noted between CKD-EPI formulae, there was variation in the range of eGFR values generated by CKD-EPI CysC (34-105 ml/min/1.73m²), CKD-EPI SCr/CysC (38-81 ml/min/1.73m²) and CKD-EPI SCr (38-67 ml/min/1.73m²).

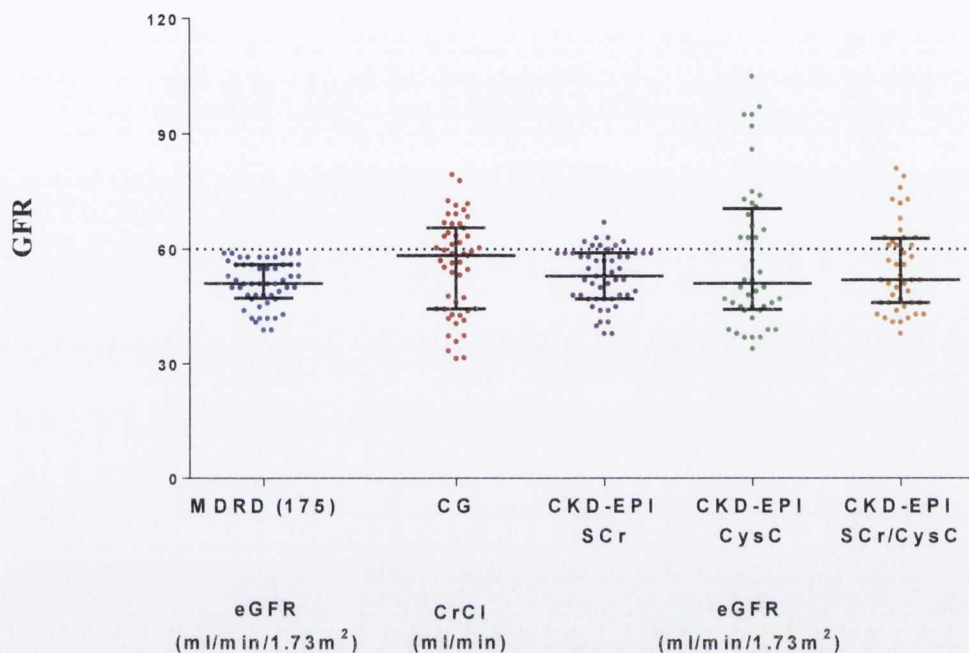


Figure 6.8 Comparison of MDRD at eGFR measurements below 60 ml/min/1.73m² with CG and all CKD-EPI equations in an oncology cohort. eGFR values below 60 ml/min/1.73m² as calculated by MDRD (dotted line) were similar to calculations using CG and CKD-EPI. Total number of patients, n=17; total number of samples, n=242. The dotted line signifies the 60 ml/min/1.73m² upper assessment limit for MDRD. p>0.05; Kruskal-Wallis H test. Measurements were expressed as median ± IQR.

While MDRD demonstrated similar measures to all formulae below 60 ml/min/1.73m², there was significant variation when compared with CG and CKD-EPI between 60 and 90 ml/min/1.73m² (****p<0.0001; Kruskal-Wallis H test; Figure 6.9). Post-hoc analysis indicated that MDRD was significantly different to CG and all CKD-EPI formulae (****p<0.0001).

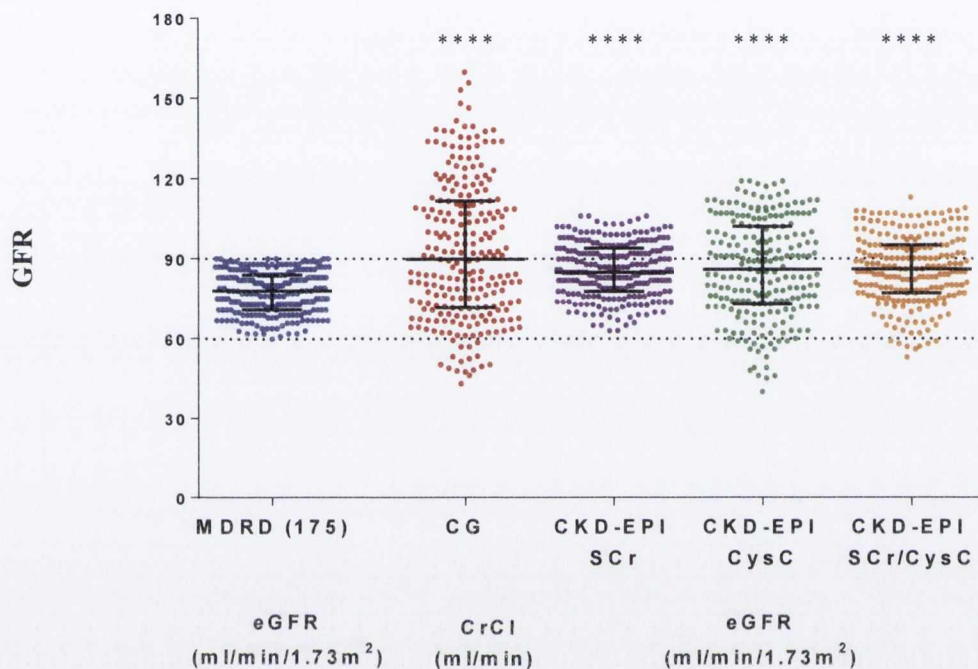


Figure 6.9 Comparison of MDRD between 60-90 ml/min/1.73m² with CG and all CKD-EPI equations in an oncology cohort. MDRD was statistically different when compared with all other formulae at eGFR measures between 60-90 ml/min/1.73m². Total number of patients, n=67; total number of samples, n=1158. The dotted line indicates the 60-90 ml/min/1.73m² assessment limits for MDRD. ****p<0.0001; Kruskal-Wallis H test, post-hoc analysis using Dunn's multiple comparison test. Measurements were expressed as median ± IQR.

Considerable variation between renal assessment formulae was also noted at eGFR values between 90 and 120 ml/min/1.73m² as calculated by MDRD (****p<0.0001; Kruskal-Wallis H test; Figure 6.10). Post-hoc analysis demonstrated differences between MDRD and CG (****p<0.0001), between CG and all CKD-EPI formulae (CKD-EPI SCr **p<0.01; CKD-EPI CysC ****p<0.0001; CKD-EPI SCr/CysC ****p<0.0001) and between CKD-EPI SCr and CysC formulae (^{δδ}p<0.01).

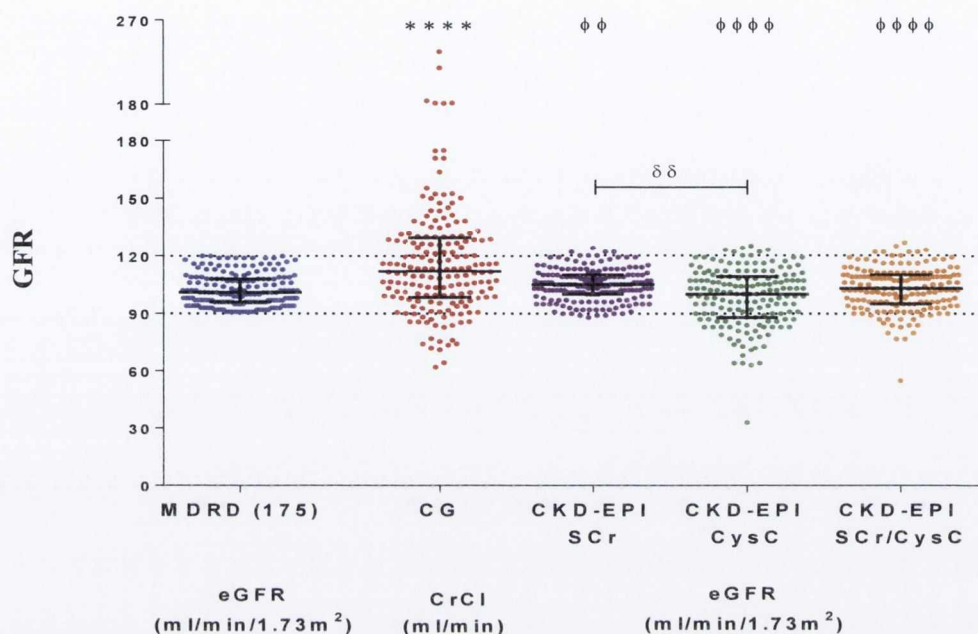


Figure 6.10 Comparison of MDRD between 90-120 ml/min/1.73m² with CG and all CKD-EPI equations in an oncology cohort. MDRD was significantly different from CG at eGFR measures between 90-120 ml/min/1.73m², while CG demonstrated discordance with all other eGFR estimating formulae. CKD-EPI SCr and CysC-based formulae were also discordant. Total number of patients, n=54; total number of samples, n=870. The dotted line indicates the 90-120 ml/min/1.73m² assessment limits for MDRD. Significant differences between MDRD and CG were represented by (*), (****p<0.0001); significant differences between CG and all other formulae were represented by (φ), (φφp<0.01; φφφφp<0.0001), significant differences between CKD-EPI formulae were represented by (δ), (δδp<0.01); Kruskal-Wallis H test, post-hoc analysis using Dunn's multiple comparison test. Measurements were expressed as median ± IQR.

In a comparison with CKD-EPI SCr, a recently proposed formula for renal assessment, no significant difference was noted at MDRD levels below 60 ml/min/1.73m² ($p>0.05$; Mann-Whitney U test; Figure 6.11.A), however eGFR measurements between 60-90 ml/min/1.73m² were significantly lower than those calculated by CKD-EPI (**** $p<0.0001$; Mann-Whitney U test; Figure 6.11.B).

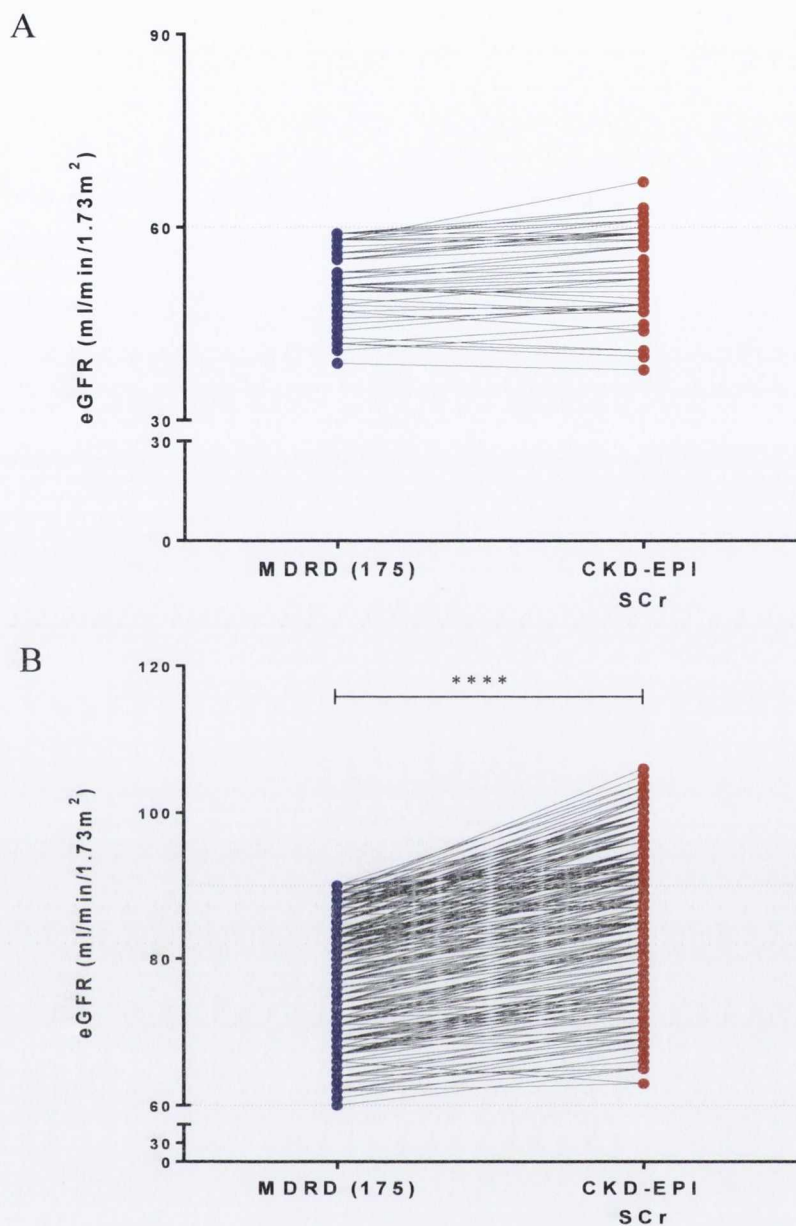


Figure 6.11 Comparison of eGFR measurements between MDRD less than 60 ml/min/1.73m² (A) and between 60-90 ml/min/1.73m² (B) and CKD-EPI SCr. Significant differences in eGFR measurements demonstrated between MDRD and CKD-EPI between 60-90 ml/min/1.73m² (dotted lines). Total number of patients, n=17 (A) and 67 (B); total number of samples, n=52 (A) and 239 (B). **p<0.0001; Mann-Whitney U test.**

6.3.7 Assessment of the CKD-EPI formulae and its application in chemotherapeutic drug dosing

Recent research has highlighted discordance when using CG in chemotherapeutic drug dosing and has indicated that improved dosing accuracy is achieved when compared with mGFR-calculated doses using CKD-EPI (Chew-Harris *et al.*, 2015). Further improvements in dosing concordance with mGFR have also been demonstrated with removal of BSA indexing from eGFR formula when it is not required to calculate chemotherapeutic drug doses such as in the case of carboplatin dosing (Dooley *et al.*, 2013). To assess the suitability of incorporating CKD-EPI into routine practice with applicability in chemotherapeutic drug dosing, CKD-EPI SCr was compared with CG, MDRD and cystatin C-based CKD-EPI formulae. CKD-EPI SCr was also compared against CG in carboplatin dosing.

In a comparison of eGFR as calculated by CKD-EPI SCr below 90 ml/min/1.73m² and other formulae, a significant difference was revealed (****p<0.0001; Kruskal-Wallis H test; Figure 6.12). Post-hoc analysis indicated a significant difference between CKD-EPI SCr and MDRD (**p<0.001) and also between MDRD and all other methods (^{δδδ}p<0.001; ^{δδδδ}p<0.0001).

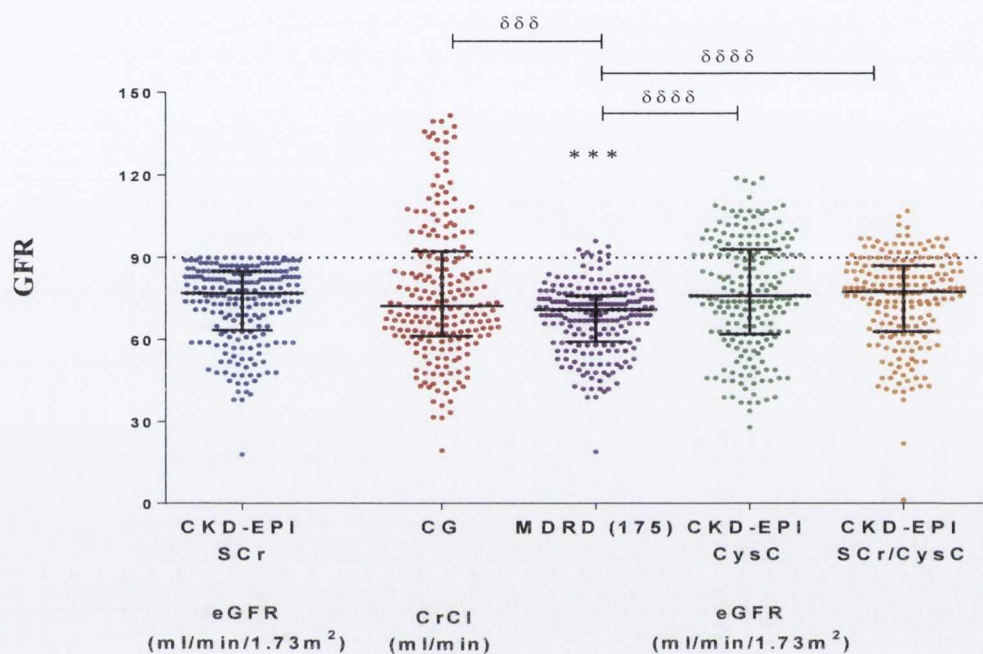


Figure 6.12 Comparison of CKD-EPI SCr with all formulae below 90 ml/min/1.73m². CKD-EPI SCr was discordant with MDRD at eGFR values below 90 ml/min/1.73m², while MDRD was also discordant with all other formulae. Total number of patients, n=46; total number of samples, n=1033. Significant differences between CKD-EPI and MDRD were represented by (*) (***)p<0.001; significant differences between MDRD and all other formulae were represented by (δ), (δδδ)p<0.001; (δδδδ)p<0.0001; Kruskal-Wallis H test, post-hoc analysis using Dunn's multiple comparison test. Measurements were expressed as median ± IQR.

Evaluation of eGFR above 90 ml/min/1.73m² as calculated using CKD-EPI SCr also revealed significant difference between formulae (****p<0.0001; Kruskal-Wallis H test; Figure 6.13). Post-hoc analysis demonstrated differences between CKD-EPI SCr and all formulae with the exception of CKD-EPI SCr/CysC (*p<0.05; **p<0.01; ****p<0.0001) and between CG and all other formulae (p<0.0001).

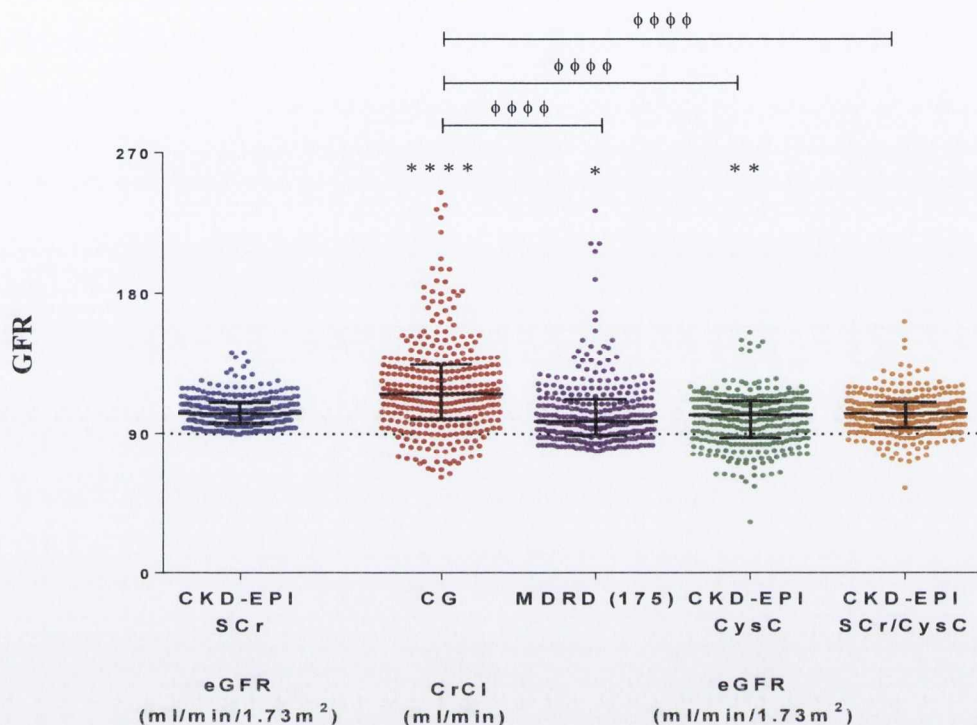


Figure 6.13 Comparison of CKD-EPI SCr with all formulae above 90 ml/min/1.73m². CKD-EPI SCr was discordant at eGFR measures above 90 ml/min/1.73m² with CG, MDRD and CKD-EPI CysC, while CG was also discordant with all other formulae. Total number of patients, n=66; total number of samples, n=1515. Significant differences between CKD-EPI and all other formulae were represented by (*), (*p<0.05; **p<0.01; ****p<0.0001); significant differences between CG and all other formulae were represented by (φ), (φφφφp<0.0001); Kruskal-Wallis H test, post-hoc analysis using Dunn's multiple comparison test. Measurements were expressed as median ± IQR.

Despite inherent inaccuracies associated with its use, CG is still used in the Calvert formula in pharmacy departments to calculate doses of carboplatin. When compared with CKD-EPI SCr at levels below 90 ml/min/1.73m², there is considerable discordance demonstrated by CG (Figure 6.14).

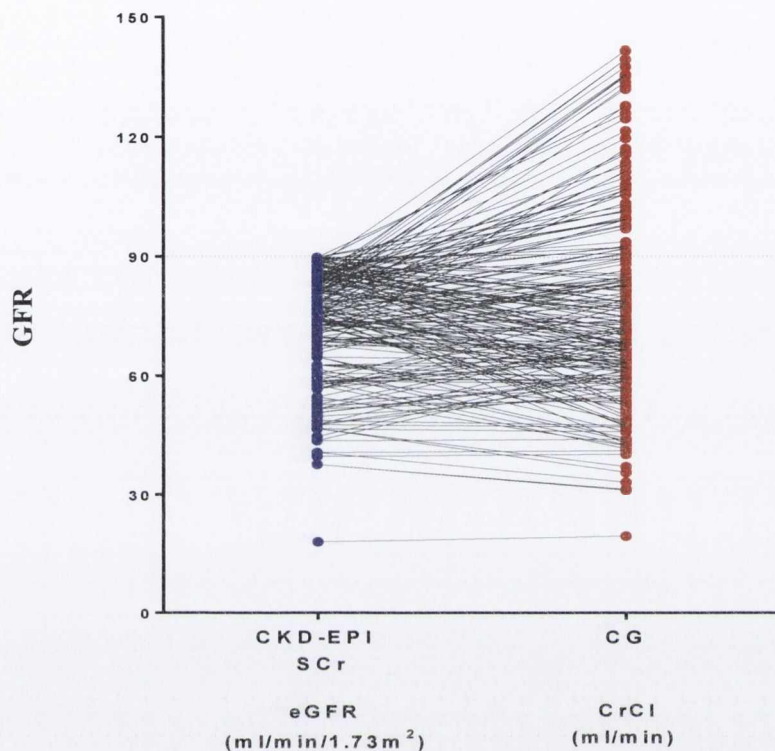


Figure 6.14 Comparison of CKD-EPI SCr and CG method at eGFR levels less than 90 ml/min/1.73m². Significant discordance between individual eGFR measures as calculated by CKD-EPI SCr below 90 ml/min/1.73m² when compared with CG (dotted line). Total number of patients, n=46; total number of samples, n=212. The dashed line indicates the 90 ml/min/1.73m² assessment limit for CKD-EPI SCr. p>0.05; Mann-Whitney U test.

To evaluate the implications of discordance in the context of chemotherapeutic drug dosing, carboplatin doses calculated by CG (values obtained from all patients between 60-99 ml/min) were compared using Bland-Altman plots with doses which would be calculated by CKD-EPI SCr with and without BSA indexing, as removal of indexing in CKD-EPI SCr calculations is thought to improve dosing concordance with gold standard mGFR-calculated doses where BSA is not used in dosing calculations (Dooley *et al.*, 2013) (using the Calvert formula with an AUC of 5, with CKD-EPI doses capped at 625 mg). With maintenance of BSA indexing in the CKD-EPI SCr formula, Bland-Altman analysis revealed 95% limits of agreement from -135.5 to 99.24 (where doses exceeding the maximum limit of 625 mg were capped at 625 mg; Figure 6.15). An 18% negative bias was observed between methods ($SD \pm 59.89$).

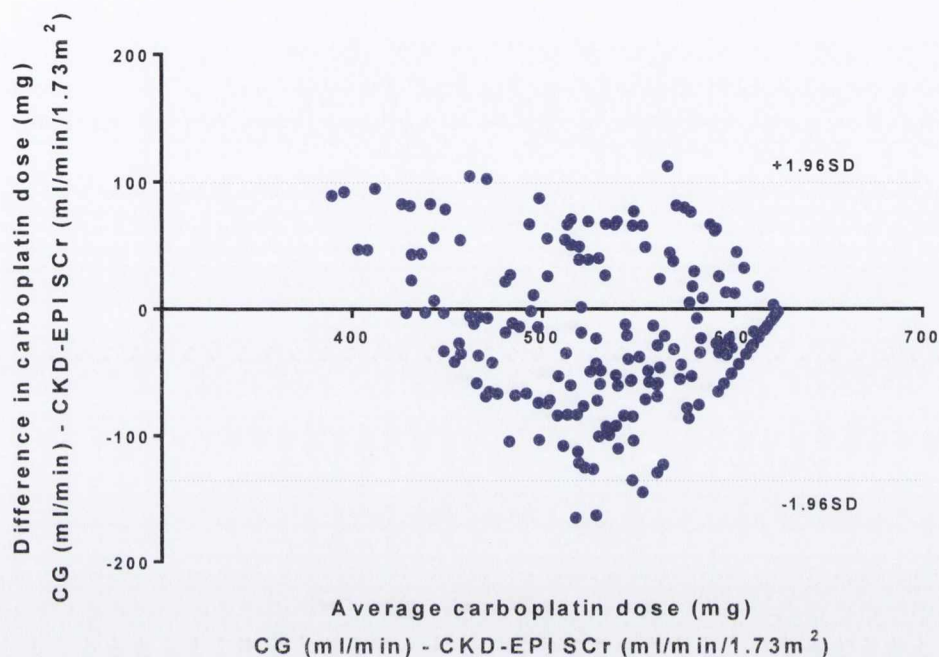


Figure 6.15 Bland-Altman plots of carboplatin dosing by CG (60-99 ml/min) and CKD-EPI SCr with BSA indexing. Significant discordance in carboplatin dose as calculated by CG and CKD-EPI SCr. Total number of patients, $n=42$; total number of samples, $n=205$. Doses of carboplatin calculated using CKD-EPI SCr which exceeded the 625 mg maximum dosing limit were capped at 625 mg, as per routine pharmacy practice. The dotted lines indicate the 95% limits of agreement between the doses calculated by both methods as measured by Bland-Altman plots (-135.5 to 99.24).

When BSA indexing was removed from the CKD-EPI SCr formula, Bland-Altman analysis revealed 95% limits of agreement from -72.44 to 64.70 (where doses calculated by CKD-EPI SCr exceeding the maximum limit of 625 mg were capped at 625 mg; Figure 6.16). A 4% negative bias was observed between methods ($SD \pm 34.98$).

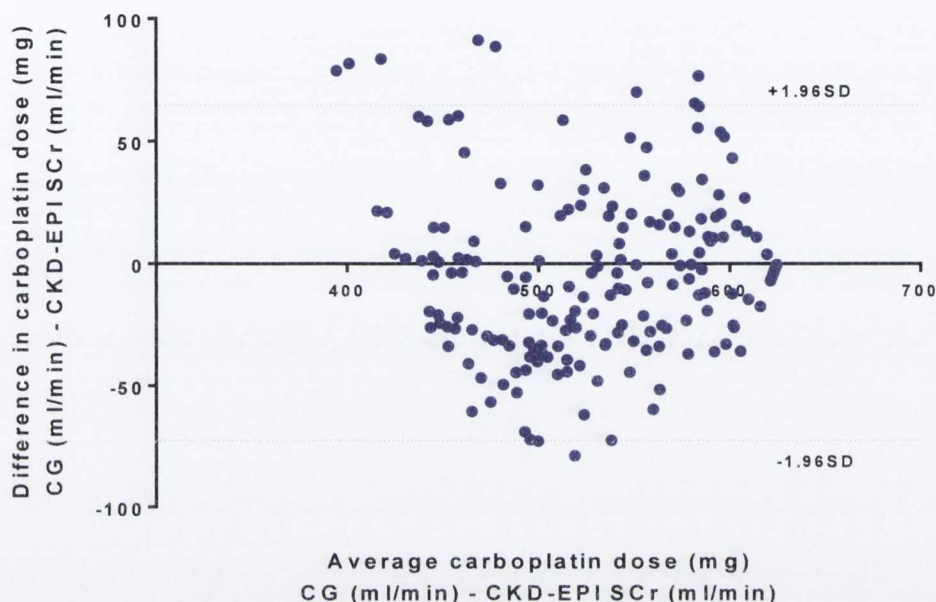


Figure 6.16 Bland-Altman plots of carboplatin dosing by CG (60-99 ml/min) and CKD-EPI SCr with removal of BSA indexing. Significant levels of discordance in carboplatin doses calculated using CG and CKD-EPI SCr when BSA indexing is removed. Total number of patients, n=42; total number of samples, n=205. Doses of carboplatin calculated using CKD-EPI SCr which exceeded the 625 mg maximum dosing limit were capped at 625 mg, in accordance with current pharmacy practice. The dashed lines indicate the 95% limits of agreement between the doses calculated by both methods as measured by Bland-Altman plots (-72.44 to 64.70).

6.3.8 Comparison of CKD staging using CG and MDRD formulae with CKD-EPI SCr

In an evaluation of CKD staging using CG and MDRD, owing to the discordance between the formulae, significant stage reclassification was observed when compared with classification using CKD-EPI SCr. When CG was compared with CKD-EPI SCr, 67 out of a total of 521 measurements were reassigned to a higher CKD stage (G1-G2, number of measures, n=50; G2-G3a, number of measures, n=16; G2-G3b, number of measures, n=1), with 77 measurements reassigned to a lower CKD stage (G3b-G3a, number of measures, n=6; G3b-G2, number of measurements, n=4; G3a-G2, number of measurements, n=18; G2-G1, number of measures, n=49). In contrast, only 5 out of 524 measurements using MDRD were reclassified to a higher CKD stage when CKD-EPI SCr was utilised (G1-G2, number of measures, n=4; G3a-G3b, number of measures, n=1), however a total of 101 measurements were reclassified to a lower CKD stage (G3b-G3a, number of measures, n=3; G3a-G2, number of measures, n=9; G2-G1, number of measures, n=89).

6.3.9 Assessment of renal function in the oncology cohort

In an assessment of patient renal function by means of evaluating eGFR values using CKD-EPI SCr, one patient (male, anthracycline cohort) consistently presented with eGFR measurements below $60 \text{ ml/min/1.73m}^2$ throughout the course of chemotherapy and was diagnosed with CKD, which was classified as Stage 4 prior to commencing chemotherapy and Stage 3 during the course of treatment (alternating between G3a and G3b) using the 2012 KDIGO guidelines. Nine patients were classified as having AKD during the course of chemotherapy, presenting with eGFR measures below $60 \text{ ml/min/1.73m}^2$. Two of these patients finished chemotherapy with improved eGFR above $60 \text{ ml/min/1.73m}^2$.

Carboplatin is recognised as one of the most nephrotoxic agents of the chemotherapeutic spectrum and as such requires close assessment of renal function during the course of chemotherapy, to minimise the risk of renal injury to

patients. In an assessment of renal function in patients treated with carboplatin (number of patients, n=22) there was no significant difference in eGFR measures throughout the course of chemotherapy ($p>0.05$; Friedman test).

In an evaluation of the presence of AKD in this patient group, a total of four patients receiving carboplatin-based chemotherapy were identified with an eGFR below 60 ml/min/1.73m² during the course of chemotherapy, indicative of AKD. All four patients were noted to have an eGFR above 60 ml/min/1.73m² prior to commencement of chemotherapy.

6.3.10 NGAL in an oncology cohort prior to commencement of chemotherapy

Elevated concentrations of NGAL have accurately facilitated detection of AKI in many patient populations, including those in the emergency department (Nickolas *et al.*, 2008; Soto *et al.*, 2013) and intensive care unit (Makris *et al.*, 2009). In this study, female oncology patients were compared with a normal female population and demonstrated significantly lower levels of NGAL prior to treatment ($p<0.05$; Mann-Whitney U test; Figure 6.17.A). This contrasted with male oncology patients who demonstrated significantly higher levels of NGAL when compared with a normal male population, however male patient numbers were small ($p<0.05$; Mann-Whitney U test; Figure 6.17.B.).

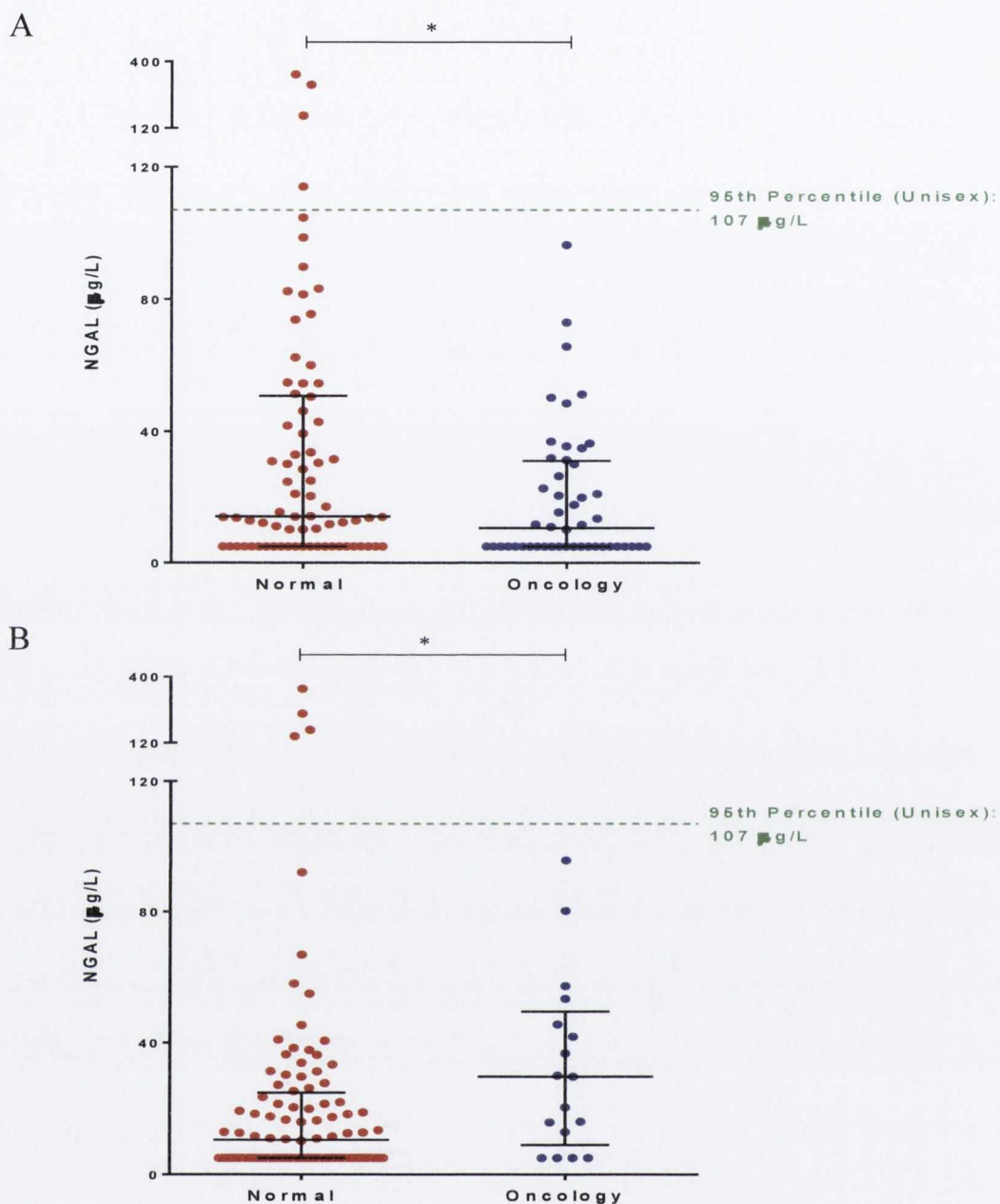


Figure 6.17 Comparison of NGAL in females (A) and males (B) of normal and oncology populations. Significantly lower concentrations of NGAL in female patients contrast with the significantly higher concentrations in a male oncology patients when compared with normal female and male populations. Total number of samples, n=122 (A) and 117 (B); total number of samples, n=122 (A) and 117 (B). * $p < 0.05$; Mann-Whitney U test. Results were presented as median $\pm$ IQR.

6.3.11 NGAL during the course of chemotherapy

To investigate evidence of AKI during the course of chemotherapy, NGAL levels were assessed in oncology patients prior to and during the course of chemotherapy. No significant difference in NGAL measures was recorded in oncology patients during the course of treatment ($p>0.05$; Friedman test; Figure 6.18). No patient demonstrated evidence of AKI. One female patient recorded a mid-treatment measurement of $174.4\text{ }\mu\text{g/L}$, which was accompanied by a decrease in eGFR as measured by MDRD and CKD-EPI of 22 ml/min/1.73m^2 respectively from pre-chemotherapy levels.

In an assessment of patients receiving the nephrotoxic agent carboplatin, no significant changes in NGAL measures were observed during chemotherapy ($p>0.05$; Friedman test), while NGAL levels were not significantly different when compared with patients receiving other chemotherapeutic regimen ($p>0.05$; Mann-Whitney U test).

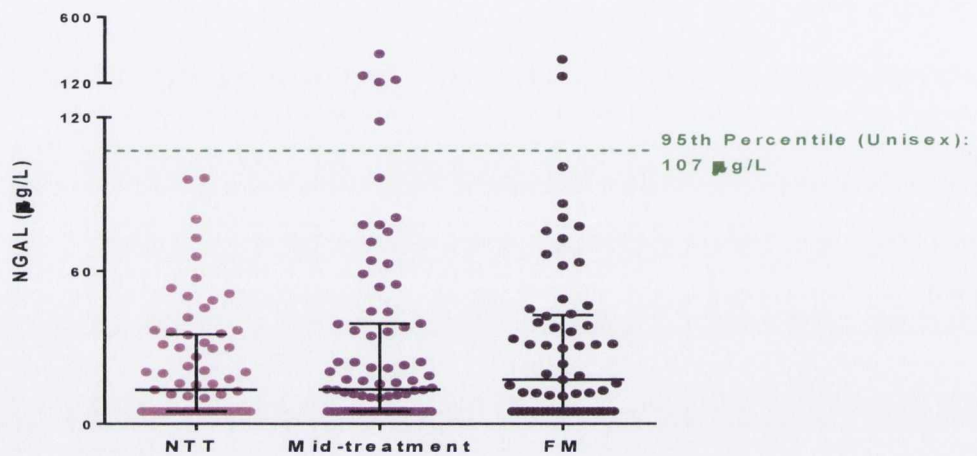


Figure 6.18 Measurement of NGAL during chemotherapy in the oncology cohort. Stable measures of NGAL demonstrated by the oncology cohort during the course of chemotherapy. Total number of patients, $n=78$; total number of samples, $n=204$. $p>0.05$; Friedman test. Results were presented as median $\pm$ IQR. NTT, naïve to treatment; FM, final measurement.

6.4 Discussion

The evaluation of cystatin C, NGAL and GFR estimating formulae to assess renal function in a cohort of oncology patients did not demonstrate a suitable alternative to creatinine measurement and has revealed considerable differences between eGFR formulae currently utilised. The findings from this study question the applicability of cystatin C in oncology patients owing to the significantly elevated levels identified in this cohort prior to and during the course of chemotherapy, thereby raising the possibility of a malignancy-mediated effect, which undoubtedly compromises its utility in eGFR equations. Furthermore, this study details evidence of disharmony between the currently utilised CG and MDRD formulae when compared with the widely promoted CKD-EPI formulae. The variability in measurements of CG-CrCl when compared with CKD-EPI SCr in chemotherapeutic drug dosing, believed to be most closely aligned to gold-standard techniques, indicated that optimum efficacy in chemotherapeutic agent dosing is not being achieved. The evaluation of NGAL was limited in this assessment, as cases of AKI were not diagnosed in this cohort. The evidence presented in this study favours the retention of creatinine measurement and strongly supports the adoption of CKD-EPI SCr as the sole formula in pharmacy and laboratory protocols for oncology patients.

Creatinine concentrations in this oncology cohort were generally comparable with a normal population prior to and during the course of chemotherapy, which strongly suggests that malignancy and treatment have no direct influence on creatinine levels. The findings in this cohort, together with stability in muscle mass which was inferred from relatively consistent patient weights recorded during the course of treatment, highlight the suitability of creatinine measurement in this patient population. However, continual assessment of factors which affect creatinine levels such as changes in muscle mass (Baxmann *et al.*, 2008), in addition to monitoring for evidence of cachexia in this patient population (Argiles *et al.*, 2015) would also be vigorously supported.

Cystatin C measures in the oncology cohort were significantly lower in females when compared with males, corroborating findings in a normal population. This

study revealed significantly elevated levels in patients prior to and during the course of chemotherapy, particularly in breast and lung cancer patients. This concurs with elevated levels previously identified in these patient populations (Tumminello *et al.*, 2009; Chen *et al.*, 2011) and resonates with findings observed in prostate (Tumminello *et al.*, 2009) and colorectal cancer (Kos *et al.*, 2000). Such evidence is suggestive of a malignancy-mediated increase in cystatin C in this cohort as eGFR measures during chemotherapy did not indicate significant renal impairment with no new CKD diagnoses. In addition, cystatin C levels in female patients were shown to increase significantly during the course of chemotherapy, findings which were replicated in an assessment of breast cancer patients in this cohort. These increases were suggestive of a treatment-specific effect on cystatin C levels which would further diminish the clinical utility of this biomarker. The elevated cystatin C levels in this patient cohort challenge its utility in the assessment of renal function in this population, a conclusion which resonates with published research findings regarding the use of cystatin C in oncology. In ovarian cancer patients, cystatin C demonstrated poor correlations with creatinine and eGFR as measured by MDRD during the early stages of chemotherapy (Bodnar *et al.*, 2010). Furthermore, cystatin C failed to detect decreased renal function in patients with various malignancies receiving cisplatin treatment (Kos *et al.*, 2013), discrediting its use in oncology. A study by Stabuc however, indicated the superior potential of cystatin C in eGFR assessment when compared with creatinine in oncology patients (Stabuc *et al.*, 2000). However, these comparisons were based on a 24 hour CrCl assessment, which is known to overestimate GFR and may be inaccurate if the patient sample was not collected correctly. Chew-Harris and colleagues (Chew-Harris *et al.*, 2015) noted negligible improvements in predicting GFR with cystatin C incorporation in CKD-EPI formulae, however they determined cystatin C levels in oncology patients to be similar to those of a kidney donor population. Cystatin C levels identified in this study cohort do not reflect those from the normal population in which the CKD-EPI CysC-based formulae were derived and as such, these formulae may demonstrate errors in assessment of eGFR in these patients and may not be suitable for use.

The assessment of overall performance of GFR estimating formulae in this oncology cohort during chemotherapy has revealed considerable discordance between CG, MDRD and CKD-EPI. As CG and MDRD are currently employed in pharmacy and laboratory practice respectively, the inconsistency in results between formulae will provoke inaccuracy when evaluating renal function in oncology patients and may have significant implications for chemotherapeutic drug dosing.

CG, which is currently used by many pharmacy departments in chemotherapeutic dosing protocols, differs significantly from all GFR estimating formulae, including CKD-EPI. As CKD-EPI has been acknowledged to be more closely aligned than CG with gold-standard renal assessment techniques (Michels *et al.*, 2010), the differences noted in results between both formulae strongly suggests that CG is not an accurate means of assessing renal function in an oncology cohort. CG was developed and based on creatinine measurements from male patients (Cockcroft & Gault, 1976) who had lower levels of muscle mass than a normal population (Rule *et al.*, 2009; Rule, 2010). While a correction factor for females was introduced, the potential for inaccuracy has remained instilled in the equation. While CG-CrCl has been shown to overestimate mGFR in large patient populations (Levey *et al.*, 1999), the overestimations in this study generated by CG for some patients may also be influenced by patient weight (McCullough *et al.*, 2015). The failure to accommodate the standardisation of the creatinine assay to isotope dilution mass spectrometry methods (IDMS) has been suggested to play a role in the inaccuracy of CG-based estimations of CrCl. In contrast, Rule contradicts such ideas in his statement that the regression involved in the development of the formula was independent of serum creatinine measures and as such, should not be influenced by the absence of standardisation (Rule, 2010). However, Rule acknowledges the importance of applying caution to assessing serum creatinine levels and hence CG-CrCl measures when assessing drug dosing recommendations developed prior to serum creatinine standardisation. As serum creatinine standardisation has been acknowledged in many cases to generate higher creatinine and lower CG-CrCl values when compared with standardised

creatinine measures, there may be a significant shift in results using standardised assays which may impact clinical decision making and drug dosing.

MDRD, the conventional formula for GFR estimation utilised by laboratories, displays significant variability in measurement when compared with CG and CKD-EPI in these experiments, in particular when compared with SCr-based CKD-EPI formulae. When compared with CG, used in chemotherapeutic agent dosing, there was considerable difference between both methods in this study, highlighting the fact that currently, there is significant variation between laboratory and pharmacy practice which will invariably lead to errors when assessing renal function. The evaluation of overall MDRD performance also revealed significant difference when compared with SCr-based CKD-EPI formulae which have demonstrated better agreement with mGFR (Levey *et al.*, 2009). While MDRD was found to compare well with CKD-EPI SCr in Bland-Altman assessment, particularly below 60 ml/min/1.73m², there was significant variation between both MDRD and CKD-EPI SCr at eGFR measurements between 60-90 ml/min/1.73m², with MDRD significantly underestimating eGFR in comparison to CKD-EPI. This data corroborates an extensive evaluation of both methods by Matsushita and colleagues (Matsushita *et al.*, 2012) and findings by Giavarina *et al.* where MDRD performance declines above 60 ml/min/1.73m² (Giavarina *et al.*, 2010) and systematically underestimates eGFR at higher mGFR levels (Stevens *et al.*, 2007). The fact that the MDRD study was originally developed using patients with defined CKD and not using a normal population is likely to account for the inaccuracy of the formula at higher eGFR levels (Levey *et al.*, 1999). The inaccuracy between MDRD and CKD-EPI has significant implications in oncology. Based on the findings from this study, MDRD may incorrectly diagnose patients with potentially more substantial renal impairment which may result in adjustments to chemotherapeutic doses, thereby influencing cytotoxic efficacy and patient prognosis.

While the assessment of all three CKD-EPI formulae in this oncology cohort demonstrated favourable performance, the elevated levels of cystatin C identified in these patients when compared with a normal population, would discourage the use of cystatin C-based CKD-EPI formulae. The increased variability

demonstrated by CKD-EPI CysC when compared with CKD-EPI SCr, likely mediated in-part by elevated cystatin C levels, showed significant discordance between both methods above 90 ml/min/1.73m². This finding was similar to results noted from a study of individuals evaluated in the CKD-EPI CysC external validation subset, where a greater bias with CKD-EPI CysC was noted when compared with mGFR in this range (Fan *et al.*, 2014). This suggests that elevated cystatin C levels may potentiate differences already present between serum creatinine and cystatin C-based CKD-EPI formulae. While the CKD-EPI SCr/CysC formula, which compared favourably in these experiments is reported in many studies to perform better than the single biomarker-based CKD-EPI formulae (Fan *et al.*, 2014; Bjork *et al.*, 2015), the elevated cystatin C measures noted in this study and the use of two biomarkers increases the variability in assessment which may not be suitable for use in this cohort. However, as creatinine measures in this population were similar to those from a normal population, these findings support the use of CKD-EPI SCr in oncology patients, provided appropriate monitoring of factors influencing creatinine measurement were performed to ensure precise evaluation of renal function.

Recent evidence has demonstrated that renal assessment formulae other than CG are favoured owing to their reported superiority in clinical practice, however despite the inaccuracy associated with CG, it remains frequently utilised in chemotherapeutic dosing protocols, including in Calvert formula calculations by many pharmacy departments. In this study, the considerable discordance between CG and CKD-EPI SCr formulae in oncology patients has been supported by a significant lack of concordance in carboplatin doses as calculated by both methods, with and without the addition of BSA indexing in CKD-EPI calculations. The comparison of carboplatin doses in this study reveals many cases where there is appreciable levels of underdosing by CG when compared with CKD-EPI SCr, which are similar to findings previously identified by Chew-Harris and colleagues (Chew-Harris *et al.*, 2015). Furthermore, they demonstrated superior P20 values (proportion of values within 20% of mGFR) for CKD-EPI in terms of chemotherapeutic dosing when compared with CG, which supports the suggestion that CKD-EPI SCr should be favoured as a more accurate means of

calculating chemotherapeutic drug doses. When assessing renal function in the context of drug dosing, the removal of BSA indexing was recommended in the 2011 KDIGO guidelines for drug dosing, as calculations are typically based on body size (Matzke *et al.*, 2011). These comments by Matzke were supported by Levey in the context of calculating drug doses for adults with acute and chronic kidney disease (Levey *et al.*, 2015). While some chemotherapeutic agents, such as doxorubicin and cyclophosphamide are dosed according to a patient's BSA, doses for agents including carboplatin are instead calculated using an AUC value. In keeping with the recommendations by Matzke and Levey, in cases where BSA is not required to calculate chemotherapeutic drug doses, we would suggest it is removed from any eGFR calculations for drug dosing. An improvement in carboplatin dosing concordance with removal of BSA indexing from CKD-EPI calculations has previously been demonstrated in a study by Dooley and colleagues, where its removal achieved a 10% increase in the number of doses which were within 20% of the mGFR calculated dose (Dooley *et al.*, 2013). While the negative bias demonstrated in this study by CG in the calculation of carboplatin doses decreased when BSA indexing was removed from the CKD-EPI SCr calculations, the results consistently support the potential for appreciable levels of underdosing by CG. Notwithstanding the improved dosing concordance which could be achieved by utilising CKD-EPI SCr in the practice of chemotherapeutic agent dosing, CKD-EPI has also been advocated in the guidance of antithrombotic and antiplatelet drug dosing in patients receiving percutaneous coronary intervention (PCI) (Parsh *et al.*, 2015). When coupled with the findings from this study, the current approach to carboplatin dosing, which utilises CG is considerably outdated and substantially inaccurate, the implications of which include lower chemotherapeutic efficacy and a potential compromise of patient prognosis. These experiments promote the consolidation of current pharmacy and laboratory renal assessment protocols and support the use of CKD-EPI SCr to improve chemotherapeutic dosing protocols.

The assessment of CKD staging using CG and MDRD compared with CKD-EPI in this study cohort further highlights the disagreement between formulae. The CG formula has been shown to be significantly different to all other formulae in

this study and the reclassification of 67 measurements to a higher CKD stage and 77 to a lower CKD stage highlights the lack of concordance between CG and CKD-EPI and suggests that CG is not appropriately assessing the renal function of oncology patients, based on current evidence which demonstrates that CKD-EPI is closest to mGFR assessment. A similar lack of concordance was observed by Parsh and colleagues in a study which assessed formula selection in patients undergoing percutaneous intervention (Parsh *et al.*, 2015). In a comparison of CG and CKD-EPI staging of eGFR measurements, Parsh demonstrates that 34% of patients were reclassified to a higher CKD stage when CKD-EPI is used instead of CG with 9% of patients reassigned to a lower CKD stage. While MDRD was also found to be discordant when compared with CKD-EPI in terms of assignment of CKD stage, the overwhelming majority of measures totalling almost 20% of all eGFR measures were reclassified to a lower CKD stage, reinforcing evidence in the literature that MDRD underestimates eGFR when compared with CKD-EPI. Similar findings were also observed by Parsh and colleagues where almost 12% of 19,720 patients assessed by MDRD were reclassified to a lower CKD stage using CKD-EPI, with only 4% assigned to a higher CKD stage (Parsh *et al.*, 2015). The majority of reclassification in this study from MDRD-assigned CKD stages was observed from G2 to G1, which denotes a change in renal function evaluation from mildly decreased eGFR to a normal or high eGFR by the 2012 KDIGO guidelines, which agrees very strongly with current evidence stating that MDRD accuracy declines with eGFR measures above 60 ml/min/1.73m². For the oncology patients in this study, an underestimation of renal function performance may impact clinical decision making with particular reference to drug dosing as outlined earlier, where decreases in eGFR may necessitate changes to chemotherapeutic doses, thereby introducing the risk of decreasing therapeutic efficacy in cases where dose adjustment is not actually warranted. The findings from this study of significant CKD stage reassignment are highly suggestive of the need to favour one formula to assess renal function across all clinical specialities which is accurate in its performance with minimal bias and imprecision and with close association with mGFR values.

Using the CKD-EPI SCr formula which is suggested to confer the best means of assessing renal function in this oncology cohort, no decrease in eGFR was observed during the course of chemotherapy in patients receiving carboplatin treatment ($p > 0.05$, Friedman test), although patient numbers were small. However four patients were identified with acute kidney disease, confirmed by at least one eGFR measure which was below $60 \text{ ml/min/1.73m}^2$, which suggests that carboplatin-based chemotherapy treatment mediates decreased renal function in some patients. This finding was reinforced by the fact that the eGFR measured prior to commencement of chemotherapy in all four patients was above $60 \text{ ml/min/1.73m}^2$. This study corroborates findings by Sleijfer and colleagues of decreased mGFR values during carboplatin treatment (Sleijfer *et al.*, 1989). The small number of patients receiving carboplatin treatment in our study precludes a definitive conclusion from being reached in terms of the potential nephrotoxicity of the agent, however there are indications of its potential to mediate renal injury based on the assessment of renal function in this study.

In the assessment of NGAL in this patient cohort, significant differences in NGAL levels were present between male and female patients when compared with a normal population prior to commencing chemotherapy. As NGAL levels have been reported to increase significantly with age (Cullen *et al.*, 2012), an older male oncology cohort, combined with small female and male oncology cohort sizes in may explain the significant differences observed between the oncology and normal populations. No patient demonstrated evidence of AKI during the course of this study, a finding which was corroborated by NGAL assessment which did not provide any conclusive indications of renal impairment or AKI in this patient population during the course of chemotherapy. No significant changes were observed in female or male patients during the sampling time points assessed in this study and for the vast majority of measurements, concentrations were considerably below the 95th percentile for a normal reference population (Cullen *et al.*, 2012). In the case of a small proportion of elevated measures which were detected, such results may correlate to the presence of leukocyturia. Previous research has alluded to the fact that this condition can mediate increases in NGAL which are not related to AKI (Cullen *et al.*, 2012),

thereby conflicting the interpretation of the biomarker. In this study, the presence of leukocyturia was not evaluated but may account for the elevated values observed. One patient demonstrated an elevated measure above the 95th percentile, which may have indicated evidence of renal impairment as it had correlated with decreasing eGFR values from baseline assessment. However, as this was a single case, further studies of NGAL in patient populations are warranted, where AKI and renal impairment are present in order to fully assess the utility of NGAL.

Chapter 7

Conclusions

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7.1 Discussion and future studies

This body of research provides an evidence base for changes in clinical practice in several areas, in particular, monitoring patients for cardiac and kidney dysfunction during and post chemotherapy. Current imaging modalities in general cannot predict subtle deterioration in cardiac function and are poor indicators in patients requiring cardiology review and management. Additionally, clinical assessment of cardiotoxicity is not always comparable across studies, with different criteria such as those published by the CREC (Seidman *et al.*, 2002; Fiuza, 2009) and the National Cancer Institute common toxicity criteria used for diagnosis (Sawaya *et al.*, 2012; Kittiwaraawut *et al.*, 2013) which makes it difficult to disseminate the full extent of cardiac injury when studies are compared. This work provides an evidence base for inclusion of hs-cTnI as a routine biomarker in patients receiving anthracycline treatment and an algorithm for its use (Figure 7.1) is presented. This is in accordance with previous NACB guidelines promoting routine cTnI measurement among patients receiving Adriamycin (doxorubicin) (Wu *et al.*, 2007). In addition to the current practice of monitoring routine liver, kidney and tumour biomarkers, the data presented here suggests that hs-cTnI using an assay with a functional sensitivity of < 5 ng/L has a clear role in monitoring these patients. Persistently elevated levels should prompt referral of those patients who require closer and more detailed monitoring with magnetic resonance imaging (MRI). Where the study was not powered to definitively determine the patients who will go on to develop cardiomyopathy and other secondary cardiac sequelae, the use of the biomarker will undoubtedly be an asset in cardio-oncologic monitoring of patients.

Furthermore, the prophylactic use of cardio-protective agents is gaining clinical credibility. These include the anthracycline cardioprotective agent dexrazoxane along with other primary prevention strategies such as β blockers, ACE inhibitors, angiotensin receptor blockers and statins (Nakamae *et al.*, 2005; Seicean *et al.*, 2012; Seicean *et al.*, 2013; Vejpongsa & Yeh, 2014). However, no definitive evidence currently exists and consensus on choice of agent is lacking. The studies to date have been limited by the lack of a real-time biomarker of change within the myocyte environment as a result of the therapy and have relied on the changes

in LV function based on ECHO, which are generally a later effect (Acar *et al.*, 2011). hs-cTnI should facilitate the identification of therapies with optimal efficacies and should be incorporated in clinical trials in the future.

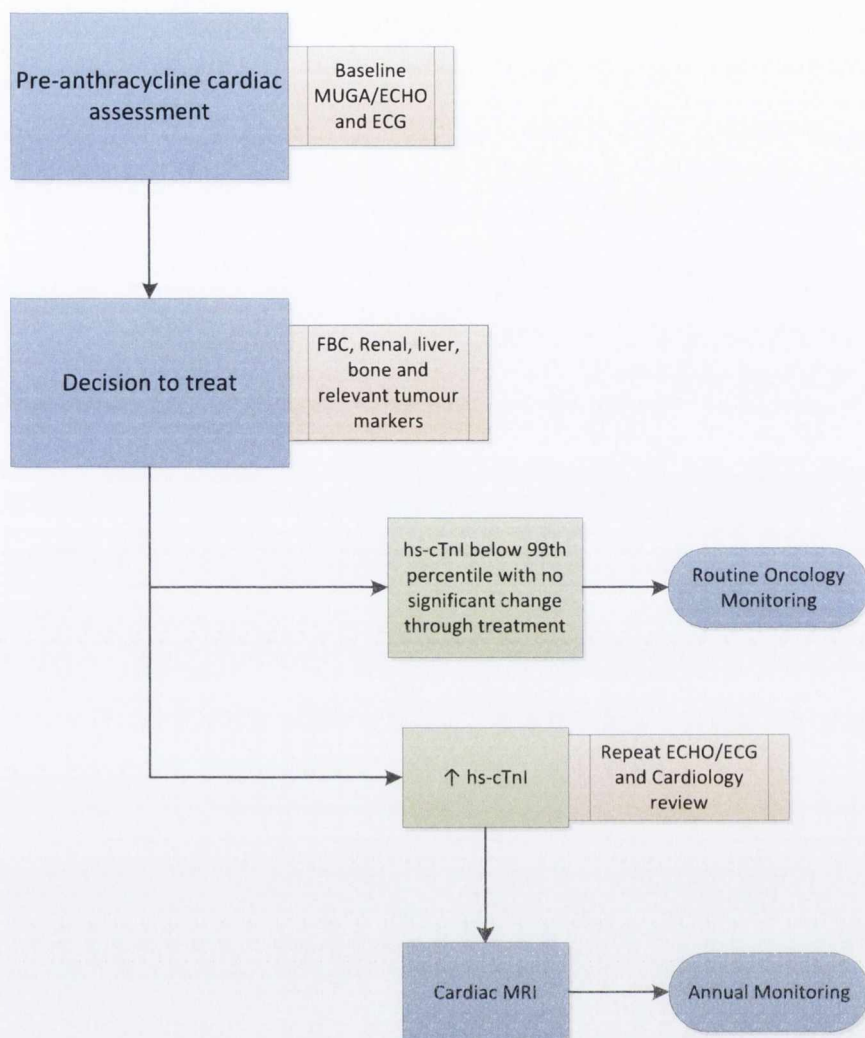


Figure 7.1 Proposed practice for patients undergoing anthracycline chemotherapy to incorporate hs-cTnI as a biomarker to guide further management.

Quantification of aldosterone, a key mineralocorticoid component of the renin-angiotensin-aldosterone pathway, is a challenging assay to perform, with most assays failing to measure aldosterone at low levels. A recent immunoassay has been validated in healthy normal subjects (O'Shea *et al.*, 2015) in this laboratory, however the assay has a functional sensitivity of 108 pmol/L, and many normal subjects have aldosterone levels below this value. A mass spectrometry method was developed and validated with the objective of achieving greater levels of sensitivity. With a functional sensitivity of 42 pmol/L, it was established that the assay is suitable for evaluating patients receiving chemotherapeutic agents without analytical interference. This has significant benefits to all patients and future work will address the evaluation of patients with potential causes of secondary hypertension. The value of a robust aldosterone assay in the oncology cohort is clinically valuable also. Some of the cardio-protective agents used in trials (Nakamae *et al.*, 2005; Akpek *et al.*, 2015) target the modulation of the renin-angiotensinogen-aldosterone pathway and the availability of this assay, will enhance the monitoring of these patients in clinical trials. A mass spectrometer with sufficient sensitivity to quantify aldosterone levels in the sub-100 pmol/L range not currently available in many centres, is required to achieve the levels of analytical sensitivity required. The data presented here is the backbone for the translation of this work into clinical diagnostics.

This study examined the biomarker galectin-3, which is indicative of cardiac fibrosis and no clinical or statistical significance was noted. A limitation of this study is that there was a short follow-up period and further longitudinal studies of patients post-chemotherapy treatment are required to validate the use of galectin-3 as an early indicator of fibrosis. This study highlights a robust assay with a potential use in predicting response to cardio-protective agents in clinical trials. In HF patients treated with rosuvastatin, low galectin-3 levels have conferred a lower risk of subsequent cardiovascular death, stroke or MI in patients when compared with those with a higher galectin-3 concentration, a finding which was even more significant when NT-proBNP measures were also low (Gullestad *et al.*, 2012). As such, galectin-3, potentially in combination with NT-proBNP may offer the potential to identify patients most likely to achieve the greatest response from

statins, which have demonstrated efficacy in cardioprotection from anthracycline-mediated cardiac injury (Seicean *et al.*, 2012; Henninger *et al.*, 2015).

Kidney renograms and mGFR are not practical for most patients and are not performed routinely in most oncology centres. Classed as “gold” standard, several authors (Hsu & Bansal, 2011; Seegmiller *et al.*, 2015) have reported clinically discordant results using these methods to assess GFR.

Clearance of an endogenous product is a surrogate for glomerular filtration rate. This study evaluated the incorporation of creatinine and cystatin C into estimating GFR. There are a number of analytical and patient-related confounders in these mathematical estimations of GFR, which particularly affect oncology patients. In this study patients were monitored through each cycle of treatment and confounders were minimised. Analytical confounders include assay interferences and calibration with international traceable material, which is now accepted practice for both creatinine and cystatin C. Up to recently (2011), cystatin C was not a standardised immunoturbidometric assay and earlier studies show significant analytical discordance, as a result of which it is flawed to translate findings from these studies. Key changes in our routine assessment of oncology patients were identified, particularly the replacement of eGFR using MDRD and CG with the CKD-EPI derivation. CG was derived before the creatinine assay was standardised and cannot be re-expressed with the new assay. Despite this knowledge the most commonly used estimation of creatinine clearance in cancer patients for determination of renal status and drug dosing is CG estimates of CrCl. This study highlights the significant clinical decisions that are based on imperfect mathematical derivations using deficient laboratory assays. Statistically significant differences found using these formulae are translated into clinical significance in terms of miss classification of oncology patients to different stages of CKD and in under-dosing of cisplatin and carboplatin agents. This study supports the evidence base from large meta-analyses where CKD-EPI was found to be the optimal estimating GFR when correlated with measured GFR. However, it is difficult to evaluate if under-dosing corresponds with poorer clinical outcome the dose of cisplatin, carboplatin is not the only treatment that these patients receive, and many are in trials of novel clinical agents. Larger studies with

homogeneous patient cohorts are required to correlate dosing levels with drug efficacy and clinical outcome. Of particular significance is the fact that the findings in this study have further implications for patients outside of oncology, in that many drug doses, including aminoglycosides, novel anti-coagulant drugs and others, are determined using mathematically derived eGFR, which we now know to be inaccurate assessments of true GFR.

7.2 Study limitations

One of the limitations of this study was the small cohort size, which limits a thorough evaluation of the biomarkers assessed. In addition, this cohort demonstrated heterogeneity in the diversity of tumour types observed, which was also reflected in the number of chemotherapeutic agents and regimens utilised. Patients in the anthracycline group were significantly younger than those in the non-anthracycline cohort, but differences in age, gender and cancer type have not been controlled for. However it is unlikely that any of these differences would significantly influence the changes in hs-cTnI observed in this study. This study was not powered to assess the levels of hs-cTnI or other cardiac biomarkers which were linked to the development of long-term or irreversible cardiotoxicity but rather emphasises that increases were indicative of subclinical cardiac injury which warrants further investigation and treatment. Furthermore, it was not possible to assess peak hs-cTnI levels occurring following completion of anthracycline administration as most patients were day-cases and could not be evaluated until commencement of the next chemotherapeutic cycle, which thereby resulted in the assessment of hs-cTnI levels at trough levels.

The gold-standard technique of measuring GFR was not possible in this study on account of the fact that its routine use in oncology is not easily achievable. The objective of this study however was not to determine the most accurate technique in this patient cohort but rather to compare and to evaluate significant differences between estimating formulae currently used. In the assessment of cystatin C, this study had limitations which included the small number of male patients recruited as well as insufficient patient numbers to specifically examine malignancy-specific characteristics. In the assessment of NGAL, patient urine samples were

not assessed for leukocyturia at time of collection. This could have explained elevated NGAL levels which did not correlate with AKI, creatinine or eGFR assessments.

Chapter 8

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IX. Appendix I

List of publications

Jones M, O’Gorman P, Kelly C, Mahon C, Fitzgibbon, M. (2016 *in press*)

High sensitive cardiac troponin-I facilitates timely detection of subclinical anthracycline-mediated cardiac injury. *Annals of Clinical Biochemistry*.

X. Appendix II

Renal Assessment formulae

$$\text{Estimated CrCl (ml/min)} = \frac{\{(140 - \text{Age}) \times (\text{Weight}) \times (0.85 \text{ if Female})\}}{(72 \times \text{SCr})}$$

Cockcroft-Gault Formula

Figure 1 Cockcroft-Gault formula used to estimate creatinine clearance (CrCl). Age, years; Weight, kg; SCr, mg/dL. (SI conversion; multiply SCr (mg/dL) by 88.4 to convert to μmol/L).

$$\text{Carboplatin dose (mg)} = (\text{AUC}) \times (\text{CrCl} + 25)$$

Calvert Formula

Figure 2 Calvert formula used to estimate carboplatin drug dose. AUC, area under the curve; CrCl, creatinine clearance.

$$eGFR = (175) \times (SCr^{-1.154}) \times (Age^{-0.203}) \times (1.212 \text{ if Black}) \times (0.742 \text{ if Female})$$

MDRD Formula

Figure 3 MDRD formula used to estimate glomerular filtration rate (eGFR). SCr, mg/dL; Age, years. (SI conversion; multiply SCr (mg/dL) by 88.4 to convert to μmol/L).

Serum Creatinine (mg/dL)		
Female	≤0.7	eGFR = 144 x SCr/0.7 ^{-0.329} x 0.993 ^{Age} [x 1.159 if black]
	>0.7	eGFR = 144 x SCr/0.7 ^{-1.209} x 0.993 ^{Age} [x 1.159 if black]
Male	≤0.9	eGFR = 141 x SCr/0.9 ^{-0.411} x 0.993 ^{Age} [x 1.159 if black]
	>0.9	eGFR = 141 x SCr/0.9 ^{-1.209} x 0.993 ^{Age} [x 1.159 if black]

CKD-EPI SCr Formulae

Figure 4 CKD-EPI SCr formula used to estimate glomerular filtration rate (eGFR). SCr, mg/dL; Age, years. (SI conversion; multiply SCr (mg/dL) by 88.4 to convert to μmol/L).

Serum Cystatin C (mg/L)	
Female or Male	
≤0.8	eGFR = (133) x (SCysC/0.8) ^{-0.499} x (0.996) ^{Age} [x 0.932 if black]
>0.8	eGFR = (133) x (SCysC/0.8) ^{-1.328} x (0.996) ^{Age} [x 0.932 if black]

Figure 5 CKD-EPI CysC formula used to estimate glomerular filtration rate (eGFR). SCysC, mg/L; Age, years.

	Serum Creatinine (mg/dL)	Serum Cystatin C (mg/L)	
Female	≤0.7	≤0.08	eGFR = (130) x (SCr/0.7) ^{-0.248} x (SCysC/0.8) ^{-0.375} x (0.995) ^{Age} [x 1.08 if black]
		>0.8	eGFR = (130) x (SCr/0.7) ^{-0.248} x (SCysC/0.8) ^{-0.711} x (0.995) ^{Age} [x 1.08 if black]
	>0.7	≤0.08	eGFR = (130) x (SCr/0.7) ^{-0.601} x (SCysC/0.8) ^{-0.375} x (0.995) ^{Age} [x 1.08 if black]
		>0.08	eGFR = (130) x (SCr/0.7) ^{-0.601} x (SCysC/0.8) ^{-0.711} x (0.995) ^{Age} [x 1.08 if black]
Male	≤0.7	≤0.08	eGFR = (135) x (SCr/0.9) ^{-0.207} x (SCysC/0.8) ^{-0.375} x (0.995) ^{Age} [x 1.08 if black]
		>0.8	eGFR = (135) x (SCr/0.9) ^{-0.207} x (SCysC/0.8) ^{-0.711} x (0.995) ^{Age} [x 1.08 if black]
	>0.7	≤0.08	eGFR = (135) x (SCr/0.9) ^{-0.601} x (SCysC/0.8) ^{-0.375} x (0.995) ^{Age} [x 1.08 if black]
		>0.08	eGFR = (135) x (SCr/0.9) ^{-0.601} x (SCysC/0.8) ^{-0.711} x (0.995) ^{Age} [x 1.08 if black]

Figure 6 CKD-EPI SCr/CysC formula used to estimate glomerular filtration rate (eGFR). SCr, mg/dL; SCysC, mg/L; Age, years. (SI conversion; multiply SCr (mg/dL) by 88.4 to convert to μmol/L).

XX. Appendix III

List of company addresses

Abbott Diagnostics

Abbott Ireland Diagnostic
Division

Lisnamuck

Longford

Ireland

Biotage

Biotage UK Limited

Hertford

Hertfordshire

SG13 7NW

Cerilliant

Cerilliant/Sigma-Aldrich
Company Ltd

Dorset

England

United Kingdom

Eppendorf

Eppendorf UK Limited

Eppendorf House

Gateway 1000 Whittle Way

Arlington Business Park

Stevenage

SG1 2FP

England

United Kingdom

Fisher Scientific

Fisher Scientific UK Ltd

Bishop Meadow Road

Loughborough

LE11 5RG

England

United Kingdom

Golden West Biologicals

Golden West Biologicals, Inc

27625 Commerce Centre
Drive

Temecula

CA 92590

USA

GraphPad

GraphPad Software, Inc

7825 Fay Avenue

Suite 230

La Jolla

CA 92037

USA

Isosciences

1017 W 9th Avenue

King of Prussia

PA 19406

USA

Millipore

EMD Millipore UK

Millipore Ireland B.V

Tullagreen

Arrigtwohill

County Cork

Ireland

UK NEQAS

UK NEQAS

UK NEQAS Office

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Sheffield

S5 7Y2

England

United Kingdom

Phenomenex

Phenomenex Ireland

Queen's Avenue

Hurdsfield Industrial Estate

Macclesfield

Cheshire

SK10 2BN

England

United Kingdom

Roche Diagnostics

Roche Ireland Ltd.

Clarecastle

County Clare

Ireland

Sarstedt

Sarstedt AG & Co.

Sarstedtstraße 1

51588 Nümbrecht

Germany

SeraLab UK

SeraLaboratories

International Limited

West Sussex

England

United Kingdom

Sigma-Aldrich

Sigma-Aldrich Company Ltd.

Dorset

England

United Kingdom

Tecan

Tecan Group Limited

Seestrasse 103

8708 Männedorf

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Thermo Fisher

Fisher Scientific UK Limited

Bishop Meadow Road

Loughborough

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