

Development of positron emission tomography imaging methods for predicting and monitoring response to breast cancer therapy

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Declaration

I declare that the work presented in this thesis is my own and that the work of others is duly referenced.

Immunohistochemistry staining, for H&E, for *in vivo* work was provided by Mr John Latigo

Immunohistochemistry staining, for H&E, TUNEL and caspase staining, for the clinical breast study, was performed by Dr Jennifer Steel and Ms Naina Patel.

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Shairoz Merchant

Dedication

To: My Family for being my pillars of strength, but especially for my husband Rahim and my son Irfan who was born during the write-up of this PhD. I could not have done it without you.

A heartfelt and sincere thank you to my parents and to my brother Rahim. You are my backbone and my unwavering support in every step of my career and life.

Abstract

In clinical breast cancer, endocrine therapy and cytotoxic chemotherapy are the mainstay of treatment. A progesterone receptor (PR) analogue can be radiolabelled by Positron Emission Tomography (PET) to assess endocrine therapy. Molecular imaging of apoptosis biomarkers can be utilised to monitor early response to chemotherapy.

To develop a pre-clinical PR PET imaging agent, LA036, based on non-steroidal tanaproget core, was selected due to high affinity with a relative binding affinity of 140%, compared to progesterone. Cell uptake studies in various PR cell lines and blocking studies with progesterone demonstrated specificity of [^{18}F]LA036 for PR. PET imaging of T47D xenografts with [^{18}F]LA036 revealed uptake in the tumour: $\text{AUC}_{0-60}(\% \text{ID/ml})$ (area under the curve from 0-60min) $498.79 \pm 29.7\%$, muscle: $(\text{AUC}_{0-60} 423.62 \pm 26.3)$. The tracer was unstable *in vivo*, with high uptake due to metabolism noted in various organs with defluorination observed in bone.

[^{18}F]ICMT-11, an isatin analogue is a novel PET radiotracer for detection of apoptosis. Treatment of a breast cancer xenograft MDA-MB-231 with combination chemotherapy resulted in increased uptake of [^{18}F]ICMT-11 in tumour within 24 hours of treatment and sustained up to 96 hours. The corresponding $\text{NUV}_{60}(\% \text{ID/ml})$ of time-points was 0.203 (Control), 0.789 (24h), 0.832 (48h), 0.903 (72h) and 0.757 (96h). PET based voxel intensity histograms revealed an increase in voxel intensity maintained between 24 and 96h post-chemotherapy. TUNEL and caspase immunofluorescence of

tumours correlated with PET data of increased apoptosis with chemotherapy while Ki67 index correlated with reduced proliferation of the tumour.

In clinical breast cancer, [^{18}F]ICMT-11 was utilised to study the optimum time point of apoptosis following chemotherapy. Patients were imaged at baseline and 24-48h post chemotherapy, exhibited an increase in standardised uptake value (SUV) in the lymph nodes; SUV_{av} 0.39 ± 0.02 (pre), SUV_{av} 0.45 ± 0.03 (post), and SUV_{max} 0.87 ± 0.02 (pre), 1.22 ± 0.12 (post). In patients imaged at baseline and 2 weeks post chemotherapy, tumour to breast ratio (TBR) and tumour to muscle ratio (TMR) were increased. Immunohistochemistry with caspase and TUNEL labelling from breast biopsies was related to apoptosis observed in the PET data.

Endocrine therapy and chemotherapy in breast cancer treatment can therefore be monitored by utilisation of molecular imaging using PET.

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List of abbreviations

AF1	Activating function 1
AF2	Activating function 2
AML	Acute Myeloid Leukaemia
AR	Androgen receptor
ATCC	American Type Culture Collection
ATM	Ataxia Telangiectasia Mutated
AUC	Area under the curve
BCA	Bicinchoninic acid assay
CPA	Cyclophosphamide
CT	Computed Tomography
CTCL	Cutaneous T Cell Lymphoma
cDNA	Complementary Deoxyribonucleic acid
DBD	DNA binding domain
DFS	Disease free survival
DHT	Dihydrotestosterone
DLBCL	Diffuse large B cell lymphoma
DNMT	DNA methyltransferase

DNA	Deoxyribonucleic acid
E2	Oestrogen
EC50	Half maximal effective concentration
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptor
ERE	Oestrogen response element
FCS	Foetal calf serum
FDG	Fluorodeoxyglucose
FEC	5-fluorouracil, epirubicin, cyclophosphamide
FNA	Fine needle aspiration
FRT	Fraction retention time
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter isoforms
GR	Glucocorticoid receptor
H&E	Haematoxylin and eosin
HAT	Histone acetylase transferase
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2

HIF α	Hypoxia-inducible factor alpha
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
Hsp90	Heat shock protein 90
IC50	Half maximal inhibitory concentration
IGFR-1	Insulin-like growth factor 1
LBD	Ligand binding domain
MAPK	Mitogen activated protein kinas
MPA	Medroxyprogesterone acetate
mRNA	Messenger ribonucleic acid
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NCoR	Nuclear receptor co-repressor 1
NHL	Non-hodgkin's lymphoma
NUV	Normalised uptake value
OS	Overall survival
PARP	Poly ADP ribose polymerase
PET	Positron emission tomography

PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PR	Progesterone receptor
PTCL	Peripheral T cell lymphoma
PVIS	PET based voxel intensity histograms
qRT-PCR	Quantitative real-time polymerase chain reaction
RANKL	Receptor activator of nuclear factor kappa-B ligand
RBA	Relative binding affinity
RECIST	Response evaluation criteria in solid tumours
RNA	Ribonucleic acid
ROI	Region of interest
RLU	Relative light units
SAHA	Suberoylanilide hydroxamine
SAR	Structure activity relationship
SCID	Severe combined immunodeficiency
SERM	Selective oestrogen receptor modulators
SPECT	Single photon emission computed tomography
SRB	Sulforhodamine B
SUV	Standardised uptake value

TAC	Tumour activity curve
TBR	Tumour to breast ratio
TCA	Trichloroacetic acid
TMR	Tumour to muscle ratio
TNM	Tumour, Node, Metastasis
TSA	Trichostatin A
TUNEL	Terminal deoxynucleotidyl transferase (TdT)- mediated dUTP nick end labelling
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VOI	Voxel of interest
WHO	World Health Organisation

Chapter 1 Introduction

1.1 Cancer

Cancer is a term used to define diseases where uncontrolled proliferation of abnormal cells occurs. A malignant growth or tumour arising from this aberrant cell growth leads to invasion of normal tissues and spread via the blood or lymphatic system. There are over a 100 different types of cancer classified according to their origin and cell type. Cancers originating from epithelial tissue are defined as carcinomas, while cancers arising from connective tissue and mesenchymal cells are defined as sarcomas. Leukaemias arise from the bone marrow resulting from malignant transformation of cells from the haematopoietic system. Lymphomas and myelomas are cancers that develop from cells of the immune system. Central nervous system cancers affect the brain and spinal cord. (National Cancer Institute, <http://www.cancer.gov/about-cancer/what-is-cancer>, 2016).

More than 1 in 2 people born after 1960 will develop some form of cancer in their lifetime (Cancer Research UK (CRUK), <http://www.cancerresearchuk.org/health-professional/cancer-statistics#heading-Zero>, 2016). The incidence of cancer increases with age. Breast, lung, bowel and prostate cancers account for over half of new cancers diagnosed each year (CRUK, 2013) and account for almost half of cases of cancer deaths in the UK (CRUK, 2012). Cancer survival rates have improved vastly with half of patient diagnosed with cancer surviving 10 years or more (CRUK, 2010-2011). This is probably due to increased awareness of cancer screening, early detection of cancer due to sophisticated imaging methods and improved treatments.

1.2 Breast cancer and hormonal therapy

Breast cancer is the leading form of cancer in women within the western world and the most common cause of death in women, estimated to be about 460,000 worldwide in 2008 [1]. Breast cancer gene expression (mRNA) profiling has led to the classification of 5 molecular subtypes including Luminal A and Luminal B which are oestrogen receptor positive (ER+ve) while normal-like which resembles normal breast profiling is associated with poor clinical outcome. The oestrogen receptor negative (ER-ve) subtypes include human epidermal growth factor receptor-2 (HER2) enriched and basal [2]. These gene signatures along with the traditional biomarkers of ER, PR and overexpression of HER2 play an important role in therapeutic intervention and predicting clinical prognosis. Commercial genomic assays such as Oncotype Dx have been developed based on these gene signatures to tailor treatment and predict clinical outcomes. ER+ve tumours respond to hormonal therapies significantly better than ER-ve tumours and patients with ER expressing tumours tend to have improved disease free survival and improved prognosis [3]. In seeking to understand tumour heterogeneity in breast cancer, a re-classification of breast cancer has been proposed. The METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) categorised the disease into ten distinct subgroups based on genome and transcriptome analysis of copy number and gene expression data. Copy number aberrations and single nucleotide polymorphism were responsible for gene expression variation. The expression also revealed amplification of IGFR1, KRAS and EGFR and homozygous deletions of BRCA1, ATM, and RB1. Cluster analysis of gene expression identified 10 distinct subgroups with distinct clinical

outcomes [4]. The Cancer Genome Atlas Network (TCGA) classified breast cancer subtypes based on various genomic and epigenomic platforms including DNA methylation, mRNA arrays, DNA copy number arrays which resulted in the convergence of this data to the four phenotypes of breast cancer (Luminal A, Luminal B, HER2 and Basal) [5]. Despite the various molecular subtypes identified, clinical breast cancer is currently diagnosed and treated using pathological assessment and immunohistochemical staining of the receptors.

Endocrine therapies are the main targeted and first-line therapies against ER+ve tumours. Selective ER modulators (SERM), such as the partial agonist tamoxifen, competitively bind to oestrogen receptors, producing a nuclear complex that decreases ER-driven transcription. ER downregulators such as the true agonist fulvestrant bind ER, block its function and cause degradation of the receptor. Treatments such as aromatase inhibition (exemestane, letrozole or anastrozole), ovarian ablation, and luteinising hormone-releasing hormone agonists reduce the levels of oestrogen and therefore prevent ligand-activation of the oestrogen receptor (Fig 1.1).

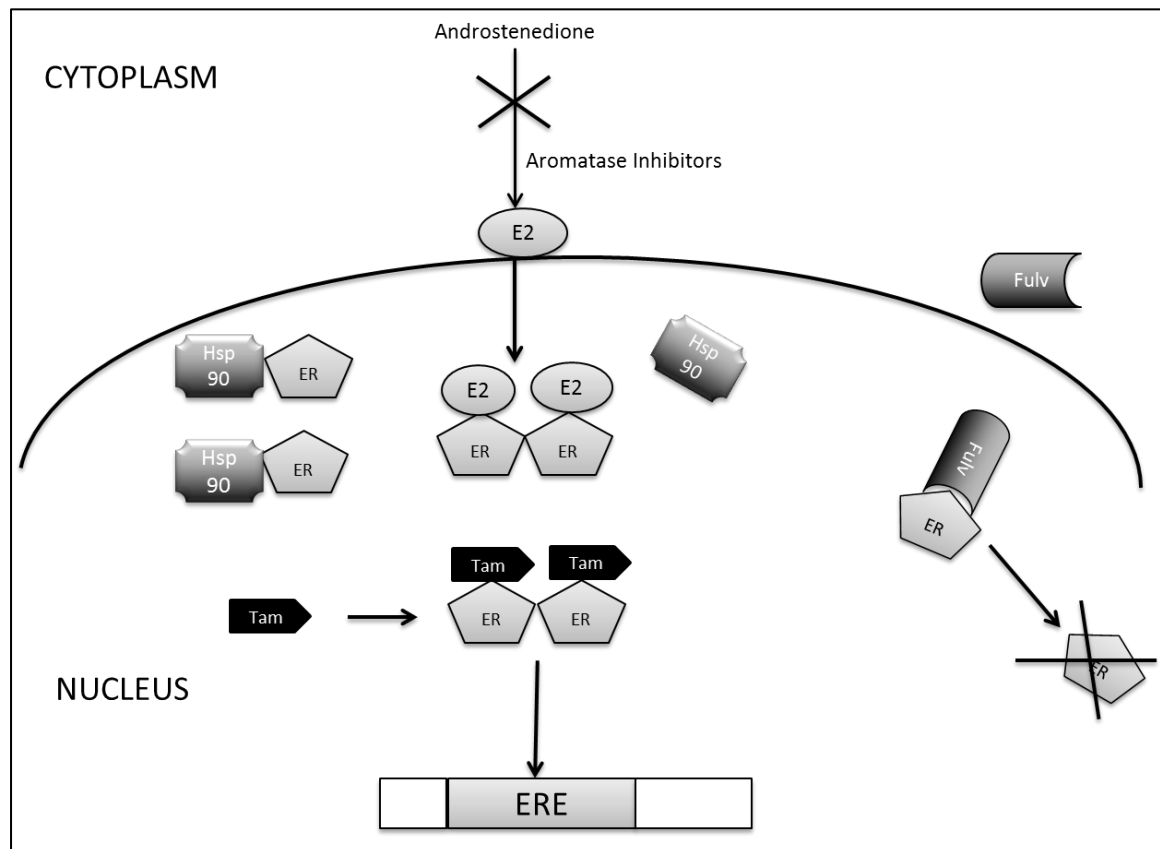


Figure 1-1: The role of anti-oestrogen treatments on oestrogen-driven transcription

ER is maintained in the nucleus by its chaperone protein Hsp90. A small proportion of ER maintained in the cytoplasm for its non-genomic action. It dimerises when ligand (either oestrogen, E2; or Tamoxifen, Tam) is present. The dimer complex translocates to the oestrogen response element (ERE) of target genes along with co-activators and repressors, resulting in gene expression (with oestrogen) or decreases DNA synthesis (with Tamoxifen). Fulvestrant (Fulv) binds ER and enables degradation. Aromatase inhibitors prevent the conversion of androstenedione to oestrogen. Abbreviations: E2, oestrogen; ER, oestrogen receptor; Tam, Tamoxifen; Fulv, Fulvestrant; ERE, oestrogen response element; Hsp90, Heat shock protein 90. (Adapted from Sharma et al, 2010)

In about 25-35% of ER+ve breast tumours, there is no response to first-line endocrine therapy or they exhibit *de novo* resistance; while some tumours that initially respond to endocrine therapy acquire resistance [6]. In metastatic tumours, approximately one third that initially respond to hormone therapy, acquire resistance to endocrine therapy and lose ER expression [7]. Enabling re-expression of ER in ER negative tumours and in tumours that have lost ER expression during relapse may provide a novel strategy for improving the efficacy of endocrine treatment for these tumour types. As a counter argument, it is possible that the ER re-expressed, may not assume the pivotal role that ER plays in breast cancer, since other mechanisms will be responsible for driving proliferation in tumour with silenced ER.

1.3 The Oestrogen receptor

The oestrogen receptor is a ligand-activated nuclear receptor that functions as a hormone dependent transcription factor, responsible for the regulation of downstream target genes that are oestrogen-responsive. In the normal breast, ER expression is restricted to epithelial cells and associated with a lack of oestrogen-regulated proliferation. In breast cancer cells, the oestrogen receptor is over-expressed in proliferating cells.

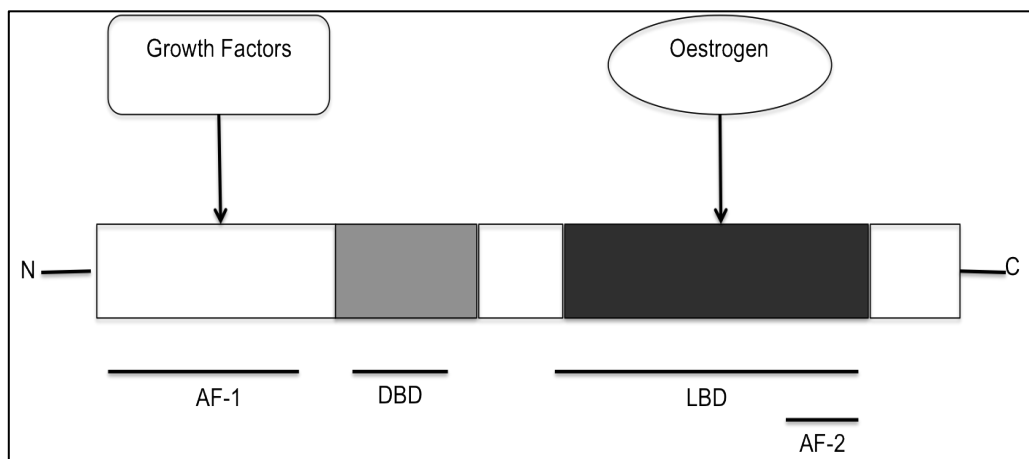


Figure 1-2: Structure of the oestrogen receptor gene

Functional domains include the DBD (DNA binding domain) and Ligand Binding Domain (LD), Activating Function 1 (AF-1) and Activating Function 2 (AF-2). (Adapted from Sharma et al, 2010)

The two oestrogen receptor isoforms, ER α and ER β , are encoded by independent genes [8]. The ER α isoform is responsible for mammary gland development and for proliferation of both normal and breast cancer cells. ER α contains two transcription activation functions AF-1 and AF-2 (Fig 1.2). The ligand-dependent activation function-2 (AF-2) binds the ligand whereas the AF-1 (activation function-1) at the N-terminus of the receptor is regulated by growth factor-induced phosphorylation [9].

The ERs are a group of proteins found within the nucleus termed nuclear oestrogen receptors and while the membrane oestrogen receptors are G-coupled proteins. [10]. In an inactivated cell, the nuclear ER associates with chaperone proteins, such as Hsp90. In the classical genomic mode of action, oestrogen (E2) binds to the ER, which allows dimerisation of the receptor [10]. Following this, the nuclear-ligand complex binds to the oestrogen response element (ERE) in the promoter region of the target gene. Co-activators such as AIB1, CBP/p300 are then recruited which enables gene transcription [11]. Corepressors like Nuclear Receptor Co-Repressor (NCoR) are associated with gene silencing. Translation leads to synthesis of proteins that are responsible for cell differentiation, growth and survival, promoting breast cancer proliferation.

In the non-classical genomic mode of action, ER can regulate gene expression by interacting with other transcription factors such as the Fos-Jun complex at other non-ERE regulatory DNA sequences such as AP-1, SP-1 [11]. In this way ER functions as a co-regulatory protein and may in addition recruit other co-activators. This mechanism of action is seen in the ER

regulation of genes such as cyclin D1, myc, BCL2 and IGF1R [11]. The non-genomic mechanism of action involves ER activation of other growth factor receptors, cellular tyrosine kinases, mitogen-activated protein kinases (MAPK), phosphatidylinositol 3 kinase and Akt signalling pathway [11]. In this mechanism, E2 activates ER in or near the cell membrane. Membrane ER interacts with growth factor signalling elements and activates these growth-signalling pathways. These kinases induce cell survival and cell proliferation through ER phosphorylation, enhancing its genomic effects. Therefore, E2 upregulates growth factor signalling which in turn activates ER. These mechanisms underpin important characteristics in understanding tumour resistance treatment strategies.

1.4 Molecular imaging.

The emergences of tumour-targeted therapies, in contrast to the conventional chemotherapy and radiotherapy treatments, have contributed to the discovery and validation of novel oncology biomarkers. A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [12].

Examples of tumour-targeted strategies include use of monoclonal antibodies such as trastuzumab targeting ERBB2 receptors in breast cancer [13] and bevacizumab antibody to vascular endothelial growth factor (VEGF) in colorectal cancer [14]. These agents have led to improved progression free and/or overall survival benefit in recent clinical trials. In addition, small molecule inhibitors of membrane bound tyrosine-kinase receptors, such as

sorafenib, a target of the VEGF receptor and raf kinase, have been developed as novel strategies for renal cancer [15]. Other examples include c-kit inhibition with imatinib in gastrointestinal stromal tumours [16] and EGFR inhibition with erlotinib in lung cancer [17]. Molecularly targeted drug therapies typically lead to cytostasis rather than cytoreduction, which occur with conventional treatments. In this setting, conventional cross-sectional imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) have limited power for the assessment of response.

Clinical CT and MRI provide anatomical information about the tumour in relation to tumour size. The criteria for response are defined by the World Health Organisation (WHO) [18] and according to the Response Evaluation Criteria in Solid Tumours (RECIST) [19, 20]. The WHO criteria requires assessment of tumour diameter in two dimensions, however, variations in WHO reporting resulted in widespread adoption of the RECIST criteria, which allows measurements of tumour in one dimension. RECIST response categories include complete response, partial response, stable disease and progressive disease. In clinical trials these imaging biomarkers define overall response and progression free survival and are currently used as surrogate endpoints of treatment, including targeted treatments [21]. However, with these novel targeted therapies, changes in tumour sizes early in treatment are minimal and may not be adequately measured according to the WHO and RECIST criteria. Novel imaging biomarkers can alternatively be used to provide information at the cellular and molecular level by non-invasively detecting changes in tumour biology such as target expression, alterations in cellular metabolism, perturbed cell signalling and other molecular

characteristics [22]. These functional/molecular imaging biomarkers fall under the umbrella term of '*Molecular Imaging*' and have the potential to diagnose, guide treatment selection, measure early response to treatment and follow disease progression.

Current modalities adapted for molecular imaging include radionuclide imaging e.g. positron emission tomography (PET) and single-photon emission computed tomography (SPECT), functional magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS), as well as ultrasound and optical imaging.

1.5 PET imaging

Positron Emission Tomography (PET) is a nuclear medicine imaging modality that provides a molecular or functional image of biological processes such as blood flow, metabolism, cell surface receptor expression, angiogenesis, hypoxia, proliferation and apoptosis. Clinically, PET-CT is used for the diagnosis and evaluation of response to treatment in cancer.

The most commonly and clinically used PET radiotracer in oncology is the glucose analogue ^{18}F -2-fluoro-2-deoxyglucose (FDG). In highly metabolically active cells such as cancerous cells, glucose uptake and utilisation, termed the 'Warburg effect' is increased. FDG is taken up by cells via facilitative transport, primarily via the glucose transporter isoforms GLUT-1 and GLUT-3 [23], and is phosphorylated by hexokinase to FDG-6-phosphate, trapping it within the cell, unlike glucose-6-phosphate which is metabolised in the glycolytic pathway. High levels of glucose transporters as well as increased

levels of hexokinase and therefore increased glucose consumption in tumours, is responsible for the high levels of FDG uptake which can be detected by PET.[24]. FDG-PET is currently used in oncology for staging, re-staging malignancy and therapy response. In FDG-PET response assessments, a significant change in FDG uptake is seen, for example, following a single cycle of chemotherapy in lymphoma [25]. While changes in FDG uptake with cytoreductive therapies may reflect loss of cell viability/cellularity, it has been hypothesised that changes in FDG uptake with a number of specific targeted therapies reflect redistribution of GLUT receptors from the plasma membrane to the cytosol consequent to the anti-cell signalling action of these drugs and subsequently by changes in glucose metabolism due to effects on gene or protein expression by the targeted treatments [26-28]. Despite wide adoption of FDG-PET in the clinical setting, FDG-PET is not effective in all tumour types and a better understanding of this heterogeneous uptake will be relevant for a better understanding of tumour biology. For example, well-differentiated prostate adenocarcinoma and renal-cell carcinoma are less metabolically active, and show low FDG uptake [29]. In contrast, more aggressive, poorly differentiated hepatocellular tumours show high FDG-uptake compared with well-differentiated tumours [30]; this is complicated by the high expression of phosphatases that can reverse phosphorylation and trapping of FDG within background normal liver together with the notion that the process of carcinogenesis is multi-stage rather than simply binary (aggressive/early stage). In tumours of the brain, a high background uptake by surrounding normal tissue can mask tumour uptake, [31] and high uptake is also noted in the presence of infected or inflamed

tissue which is a limiting factor of FDG use [32]. Furthermore, after treatment, high uptake by infiltrating immune cells can mask the decreased uptake by the dying tumour cells [33]. There is therefore an urgent need to develop novel imaging biomarkers that measure other biological processes in tumours such as proliferation, apoptosis, vasculature dynamics and receptor expression to provide readout of on-drug pharmacological effect of mechanism-based anti-cancer drugs.

1.6 Aims of the thesis

- 1) To examine a model system using epigenetics for re-expressing ER and therefore PR
- 2) To develop new imaging methods to evaluate response to commonly used breast cancer treatment such as endocrine therapy and chemotherapy
- 3) To develop a novel PR imaging agent as a marker of ER signalling and eventually to explore the response to endocrine treatment
- 4) Develop further the dynamics and determine the temporal nature of apoptosis in breast cancer xenografts using the apoptosis radiotracer ICMT-11
- 5) Explore the optimum timepoint of apoptosis using ICMT-11 apoptosis radiotracer and relation to clinical outcome for patients with breast cancer treated with neoadjuvant chemotherapy in a Phase 1b trial.

Chapter 2 Materials and Methods

2.1 Chemicals and Reagents

Estradiol, 5-aza-dC, Progesterone, Medroxyprogesterone acetate (MPA), Dexamethasone, Dihydrotestosterone (DHT), *p*-nitrophenylphosphate and TSA were obtained from Sigma Aldrich (Dorset, England). SAHA was obtained from Cayman Chemical (Ann Arbor, Michigan, USA). Monoclonal anti-ER α antibody was obtained from Leica-Microsystems (Buffalo Grove, IL, USA). Additional monoclonal ER primary antibodies, sc542, sc8002 were obtained from Santa Cruz Biotechnology (Dallas, Texas). Monoclonal PRA/B was obtained from Cell Signalling (Danvers, MA, USA). Cyclin D1, ps2 and cathepsin D and β -actin were obtained from abcam® (Cambridge, UK). Secondary antibodies, goat anti-mouse HRP and goat anti-rabbit HRP were also obtained from Santa Cruz Biotechnology. Taqman® Universal Master Mix, ER1 (alpha), PR and GAPDH primers were all obtained from Applied Biosystems (Life Technologies, USA). One-GLO and Dual-GLO luciferase assay systems were obtained from Promega (Madison, WN, USA). ABC transporter inhibitors Zosuquidar MK571 and Fungal toxin fumitremorgin (FTC) (Sigma Aldrich) were a kind gift from Dr Maciej Kaliszczak

2.2 Cell lines and culturing

MCF7, MDA-MB-231, T47D, BT474 (breast cancer cell lines) and A549 cells (lung cancer cell lines) were obtained from ATCC (American Type Culture Collection). MELN cell line (MCF ERE Luciferase reporter) were a kind gift from Dr Madge Lake [34]. LnCap (AR Luciferase reporter) cells were kindly donated by Dr Ailsa Sita-Lumsden, of Professor Charlotte Bevan group, Imperial College, London. Cell culture of MCF-7 and MELN cells was

performed in Dulbecco modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% foetal calf serum (FCS) and 100 units/ml penicillin, 100µg/ml streptomycin and 2mM glutamine. MDA-MB-231, T47D, BT474, LnCap (AR) and A549 cells were grown in RPMI-1640 supplemented with FCS, 2mM Glutamine, 100units/ml penicillin and 100µg/ml streptomycin. For culturing in oestrogen-free conditions, cells were cultured in DMEM lacking phenol red (Invitrogen) supplemented with 10% dextran-coated charcoal stripped FCS (First Link Ltd UK, Wolverhampton, UK), 2mM Glutamine and 100 units/ml penicillin, 100µg/ml streptomycin. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

2.3 Sulforhodamine (SRB) assay

An SRB assay is a method for *in vitro* cytotoxicity screening [35]. The assay is used for cell density determination, and relies on the ability of the SRB to bind to protein components of cells fixed to tissue culture plates by trichloroacetic acid (TCA). Cells were seeded in a 96 well plate at varying cell numbers from 2000 to 10000. Seeding density was selected to produce an optical density of 2-3 following vehicle treatment (i.e. the point at which exponential growth is occurring). 24 hours post seeding, the relevant drug (e.g. SAHA, 5-aza-dC) was reconstituted in appropriate solvent and then serially diluted in media to obtain a range of drug concentrations (0.1µM to 40µM, n=6 per concentration), which were added to individual wells. Each dose response experiment was repeated 3 times. 72 hours after addition of drug, the cells are fixed with 50% (TCA) for 1h, then washed 10 times with water, followed by incubation with 0.4% SRB (Sigma Aldrich, Dorset, England) for half an hour.

Following this, they were washed with 1% acetic acid and air dried. Absorbance at 540nm was determined using a microplate reader after solubilisation of dye by addition of 10mM TRIS to each well. A growth inhibition curve was used to determine the decrease in cell proliferation and determine the IC₅₀. Analysis was carried out using GraphPad Prism software, Version 5.0a (La Jolla, CA, USA).

2.4 RNA quantification and qRT-PCR analysis

Total RNA was extracted from 80% confluent 6 well plates containing MCF7 and MDA-MB-231 cells treated with vehicle, 5-aza-dC, TSA, SAHA or a combination treatment using the RNA isolation kit RNEasy (Maxi) kit (Qiagen, Hilden, Germany). Quantification of RNA was performed using the NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Walham, Massachusetts, USA). Total RNA (10ng) was reverse transcribed to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosciences). qRT-PCR was performed using the TaqMan MicroRNA assay kit (Applied Biosystems, Foster City, CA USA). The reaction mix consisted of 10 µl of TaqMan universal master mix (2x), 1 µl of TaqMan gene expression assay (including the ESR1 (Hs00174860_m1), PR (Hs01556702_m1) and control GAPDH (Hs02758991_g1) primers; ThermoScientific Fisher) 20x, 1ng of cDNA in a final volume of 20 µl. Quantitative real-time PCR (qPCR) was performed on triplicate samples with thermal cycling parameter of 95°C for 10 minutes, 40 cycles of 95°C for 15s and 60°C for 1 minute on an ABI Prism 7900HT sequence detection system (Applied Biosystems). Normalisation was performed using the delta Ct method.

2.5 Western blotting

Cells were washed with phosphate-buffered saline (PBS) and lysed with a combination of passive lysis buffer (Promega, Madison, WI, USA) and Halt Protease and Phosphatase Inhibitor Cocktail (Thermoscientific, Waltham, MA, USA). Cells lysates were placed on a rotator for thirty minutes at 4°C, followed by centrifugation at 12000g. Protein concentrations from supernatant obtained were quantified with the BCA (bicinchoninic acid assay) protein assay, according to manufacturer's instructions (Thermoscientific Fisher). After adjustment to the same total protein content, cell lysates were analysed using western blot analysis. Twenty to thirty micrograms of cell lysates were heated to 70°C for 10min with LDS sample buffer and reducing agent (Invitrogen, Carlsbad, CA, USA). Proteins were separated on NuPAGE Novex 4-12% Bis-tris gel (Invitrogen) at 200V for 50min or Mini-PROTEAN TGX Precast Gels (Bio-rad, Hercules, CA, USA) at 200V for 15min. Following electrophoresis, proteins were transferred to a PVDF membrane using a semi-dry transfer system, Transblot Turbo Transfer system (Bio-rad, Hercules, CA, USA) and then blocked with blocking buffer (1 % milk in Tris-Buffered Saline and Tween 20 TBST) for 1h. The membranes were then probed with the primary antibodies ER antibody 1:2000 (mouse), PR antibody 1:1000 (rabbit), β -actin 1:5000 (rabbit). Following overnight antibody incubation in blocking buffer, followed by 3x 10min washes in fresh blocking buffer, the PVDF membrane was incubated with peroxidase labelled relevant secondary antibodies (goat anti-mouse HRP and goat anti-rabbit HRP). The western blot reactions were detected by chemiluminescence-based photoblot system (ECL –. GE Healthcare, Chalfont St Giles, Bucks, UK).

2.6 Cell transfection and luciferase assays

For the A549 cells, transfected with Mouse Mammary Tumour Virus reporter plasmid (containing glucocorticoid receptor), cells were seeded to 70-80% confluency in clear-bottomed black 96 well plates on the day prior to transfection in antibiotic and oestrogen-free DMEM. The following day, cells in each well were treated with 200ng of plasmid, 0.6µl of Lipofectamine 2000 transfection reagent using phenol-red free DMEM media supplemented with 2mM L-glutamine. To assess for transfection efficiency and to normalize the data, 50ng of pRLTK (renilla luciferase) was added to the plasmid mixture. 6 hours following transfection, the media with transfection reagents were aspirated from the wells and replaced with normal media containing test compounds including dexamethasone, LA036 and medroxyprogesterone acetate (MPA). In stable cell lines containing androgen receptor and oestrogen response element, the transfection step was not necessitated and cells were treated with dihydrotestosterone or oestrogen in the LnCap and MELN cell lines respectively.

For assessment of luciferase activity in the transiently transfected cell line, firefly and renilla luciferase activity were measured 48h after treatment, using the Dual-Glo Luciferase assay system. Media containing test compounds was aspirated and 60µl of 1:1 phenol-red free media and luciferase reagent was added to assess luciferase activity. After 10min incubation, luciferase activity was quantified using Topcount:NXT Luminescence (Perkin Elmer, Buckinghamshire UK). To normalize for transfection efficiency and to assess

renilla luciferase activity, 30 µl of Stop and Glo reagent was added to the wells and again luciferase activity was quantified using Topcount.

For the stable cell lines, LnCap and MELN, luciferase activity was determined by the One-Glo assay 48h after treatment with test compounds. Luciferase activity was determined after addition of 50 µl of One Glo luciferase reagent and activity determined by Topcount. Data was normalized to total protein, determined by the BCA assay following luciferase activity measurement.

2.7 T47D alkaline phosphatase assay

In the high PR-expressing T47D breast cancer cell line, progestins induce *de novo* synthesis of alkaline phosphatase. The enzyme induction is specific to progestins and is utilised as a downstream reporter of PR activation by progesterone and its analogues. Alkaline phosphatase produced by the T47D cells phosphorylates the substrate *p*-nitrophenyl phosphate, resulting in a change in its optical density, measurable by colorimetry. This technique is commonly applied as a quick and cost-effective down-stream measure of PR activity. T47D cells were plated at 50,000 cells per well in 96 well plates in DMEM with 10% FCS. The following day the medium was replaced by DMEM without phenol red. 24 hours after this, the test compounds were added to the media. The cells were cultured for another 24h and tested for alkaline phosphatase by the addition of *p*-nitrophenyl phosphate. Optical density measurements were taken at 5-minute intervals for 30 minutes at a wavelength of 405nm. The results were plotted for each of the concentrations and the change in optical density over time resulted in the calculation of the EC₅₀. The amount of alkaline phosphatase-mediated phosphorylation was

proportional to progestin analogue binding and subsequent downstream activation of the PR signalling. It is of note that this assay was only sensitive for PR agonists; the assay is insensitive to small molecules that bind to PR with high affinity but act to inhibit PR (antagonist).

2.8 Fluorescence polarization assay

This competition assay was used to determine the relative binding affinity of progesterone analogues to PR. In this cell-free assay, changes in fluorescence polarization were used to determine relative PR-binding. The assay constituents included the PR ligand-binding domain fused to glutathione transferase and a fluorescently tagged progesterone ligand. The ligand, when bound to the progesterone receptor ligand-binding domain exhibited a high polarisation value following excitation of linearly polarized excitation light. In the presence of effective competitors, the ligand was displaced from the ligand-receptor complex, resulting in a low polarisation. The concentration of the test compound resulting in half-maximum shift in polarisation was the IC_{50} of the compound (Figure 2.1). The assay is utilised with agonists and antagonists of PR.

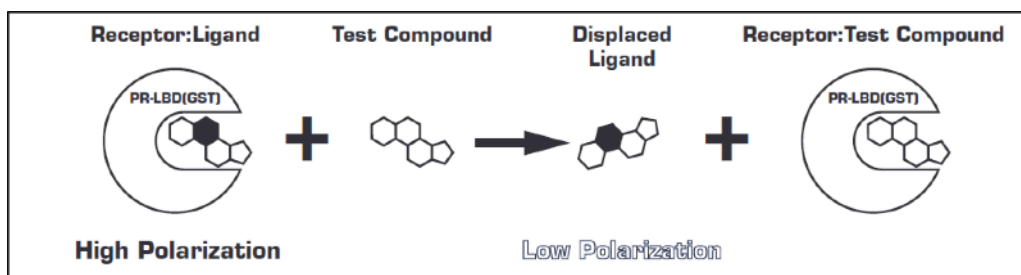


Figure 2-1: Fluorescence Polarisation assay

High polarisation is observed with the receptor-fluorescence ligand complex. In the presence of effective analogues, the fluorescent ligand is displaced and a low polarisation value is obtained (Figure from Invitrogen).

2.9 Radioactivity cell uptake assays

MCF7, MDA-MB-231, T47D and BT474 cells (1×10^5) were plated into 6 well plates in normal media and oestrogen-depleted media 24h prior to treatment. 24h later cells were either treated with vehicle or 10nM oestradiol (E2). A further 24h later, 0.74MBq [^{18}F]LA036 was added to the individual wells. For blocking studies, cells were co-incubated with 100nM-10 μM of cold progesterone compound. Cell plates were then placed in an incubator at 37°C in a humidified atmosphere of 5% CO_2 for 60min. Cells were subsequently washed 3 times with ice-cold PBS, and cells lysed with 1ml of RIPA buffer and incubated for a further 10 minutes on ice. Cells were scraped and 800 μl of cell lysate were transferred to counting tubes and decay-corrected radioactivity was determined on a gamma counter (Cobra II Auto-Gamma counter, Packard Biosciences Co, Pangbourne UK). The remaining aliquot of cell lysate was used to determine protein concentration using the BCA 96 well plate assay (Thermo Fisher Scientific Inc, Rockford, IL, USA). Radioactivity was normalised to protein content and expressed as a percent of total radioactivity per mg of protein.

2.10 Small animal experimental models for PET

All animal experiments were performed in accordance with the United Kingdom Home Office Guidance on the Operation of the Animal (Scientific Procedures) Act 1986, Amendment Regulations 2012 and within the guidelines of the UK National Cancer Research Institute Committee on Welfare of Animals in Cancer Research [36].

The *in vivo* experimental models were established in SCID beige mice (Harlan) aged 6-8 weeks for the T47D cell line and BALB/c nude mice aged 6-8 weeks (Harlan) for the MDA-MB-231 cell line. For T47D xenografts, mice were subcutaneously implanted with 0.72 mg/60 day release estradiol pellets (Innovative Research of America, Sarasota, FL) approximately 2 days before cell inoculation. Xenografts were established by subcutaneous injection of 100 μ l of T47D cells (8×10^6 cells in RPMI media mixed 1:1 with Matrigel, BD Biosciences) and MDA-MB-231 cells (5×10^6 cells in PBS) on the back of the mice. Tumour dimensions were monitored periodically using calliper measurements and tumour volumes were calculated by the equation: volume = $(\pi/6) \times a \times b \times c$ where a, b and c represent orthogonal axes of the tumour. When tumour volumes reached 80-100mm³ (T47D $\approx$ 6 weeks, MDA-MB-231 $\approx$ 2 weeks), mice were used for PET imaging for PR with T47D xenografts, while MDA-MB-231 xenografts were treated with vehicle (saline, 0.012ml/g) or combination chemotherapy 5-fluorouracil (5FU) 65mg/kg, epirubicin 10mg/kg and cyclophosphamide (FEC) 130mg/kg to obtain PET images at 24, 48, 72 and 96h time points.

For biodistribution studies, [^{18}F]LA036 (~3.7MBq) was injected into the tail vein of anaesthetized non-tumour and tumour bearing T47D mice. The mice were maintained under anaesthesia and sacrificed by exsanguination by cardiac puncture and tissues harvested (n=3 both groups). Biodistribution studies were done at 60min post injection of radioactivity to permit correlation to dynamic studies. Tissue radioactivity was determined on a γ counter (Cobra II Auto-Gamma counter) and decay-corrected. For data normalisation, 10 μ l standards from a 1:100 dilution of the stock activity were counted. Plasma was obtained by centrifugation of terminal blood samples (6,000g, 5 min). Data were expressed as percentage injected dose per gram of tissue (%ID/g).

2.11 PET imaging studies (*in vivo*)

Mice were anaesthetised using isoflurane inhalation and Dynamic [^{18}F]LA036, [^{18}F]FDG and [^{18}F]ICMT-11 imaging was carried out on a dedicated small animal PET scanner (Genisys⁴ PET/X-ray, Sofie Biosciences, Culver City, California). Imaging scans were carried out following a bolus i.v. injection of 3.7MBq of the radiotracer in the tail vein of tumour-bearing mice. Static scans were acquired for 10 min (at 50–60 min) for [^{18}F]ICMT-11 and images were reconstructed using maximum-likelihood expectation maximization (matrix size, 208 $\times$ 96 $\times$ 96). Dynamic emission scans were acquired in list mode format over 60 minutes. Data were sorted into 0.5-mm sinogram bins and 19 time frames for image reconstruction (2D-OSEM)(4 $\times$ 15s, 4 $\times$ 60s, and 11 $\times$ 300s). The Siemens Inveon Research Workplace software was used for visualisation of radiotracer uptake. 30-60min cumulative images of the dynamic data were used to define 3-dimensional regions of interest (ROI).

The count densities were averaged for all ROIs at each time point to obtain time versus radioactivity curves (TAC). Tumour TACs were normalized to injected dose measured by a VDC-304 dose calibrator (Veenstra Instruments) and normalised uptake was expressed as percentage injected dose per mL tissue (NUV; %ID/mL) or normalised to that of the whole body to obtain the normalized uptake value (NUV) for [^{18}F]ICMT-11 data. Normalised uptake of radiotracer at 60min (NUV₆₀) was used for comparisons. For qualitative image visualisation, cumulative images of the dynamic data (30–60min) were also iteratively reconstructed (OSEM3D).

2.12 PET based voxel intensity sorting histograms(PVIS)

For each tumour ROI (region of interest), voxel intensities were extracted and sorted as per their intensity frequency to give the PET based voxel intensity sorting histograms (PVIS). These were further analysed statistically using Prism v5.0 (Graphpad). A 95th percentile cut-off was chosen arbitrarily to select the highest 5% of voxels likely to represent apoptotic cells.

2.13 Immunohistochemistry and immunofluorescence

Tumours were excised, fixed in formalin, embedded in paraffin, sectioned (5 μ m slices) and processed for fluorescent detection of ER, PR (as per section 2.1) and Ki67 using primary antibodies and further incubated with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit (Invitrogen) antibodies. The ProLong Gold Antifade mounting solution (Invitrogen) containing 4',6-diamidino-2-phenylindole (DAPI) was added to tissue sections prior to coverslip mounting. Sections were also counter-stained with haematoxylin and eosin (H&E). Images were captured using an Olympus BX51 fluorescent microscope.

2.14 Protein immunoblotting of tumour samples

Tumours were snap frozen on excision following PET scanning and biodistribution. Frozen tumour tissue samples were homogenized in RIPA lysis buffer with the PreCellys 24 homogenizer and CK14 beads-containing tubes (2 cycles of 25 seconds—6,500 rpm). Equal amounts of protein (30 μ g) were denatured in sample buffer, subjected to SDS-polyacrylamide gel electrophoresis on 4% to 12% gels, and transferred to polyvinylidene fluoride membranes. The membranes were immunoblotted with ER, PR and anti-actin (β -actin) primary antibodies followed by incubation with anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies respectively, and visualised by enhanced chemiluminescence.

2.15 Statistical analysis

Statistical analysis was performed using Graphpad Prism Version 5.0a (Graphpad Software, California, USA). Unpaired t-tests was used to compare control versus treatment arms. Analysis of variance (ANOVA) was used for large data sets. Bonferroni or Dunnett's post tests were used to compare all treatments and vehicle versus treatment, respectively.

Chapter 3 The role of epigenetics in the re-expression of the silenced oestrogen receptor

3.1 Histone acetylation and deacetylation

Nucleosomes are the repeating units of chromatin and allow DNA to be tightly packed into a cell nucleus. Nucleosomes are then folded to eventually form a chromosome [37]. A nucleosome is defined as a complex that consists of DNA wrapped around eight small proteins called histones (two molecules each of histone H2A, H2B, H3 and H4). 146 base pairs of DNA bind the nucleosome whilst 50-70bp of linker DNA is bound by a linker histone H1 which separates each nucleosome [37]. Each core histone has a structured domain and an unstructured amino-terminal tail which can be subject to enzyme modification by post-translational acetylation, methylation and phosphorylation [38]. Histone modification is important for transcriptional regulation. Histone acetylation of lysine residues, characterised by histone acetyl transferases or HATs, weakens DNA-histone interaction allowing DNA to be accessible for transcription. Conversely, histone deacetylation (catalysed by histone deacetylators or HDACs) is associated with gene silencing (Fig 3.1). Histone acetyltransferase (HAT) and Histone deacetylases (HDAC) are important components of the ER transcriptional complex. HDAC1 can suppress ER transcriptional activity by interacting with AF-1 and the DBD domains.

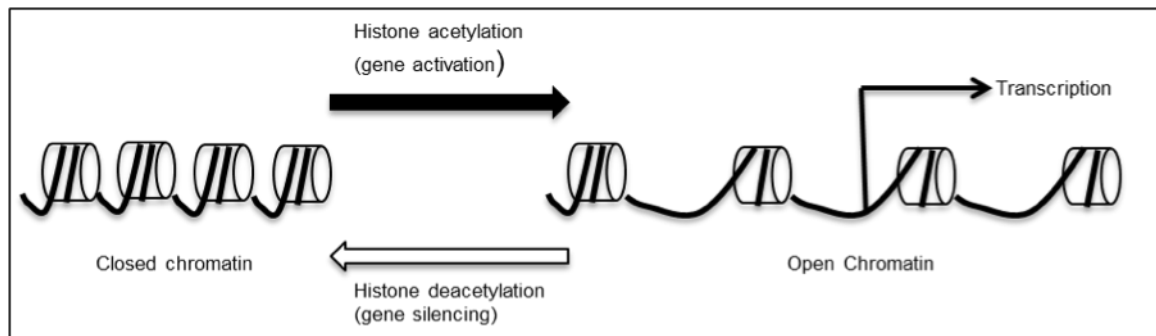


Figure 3-1: Histone acetylation and deacetylation in gene expression

Histone acetylation allows for open chromatin and enables gene expression while gene silencing is as a result of tight chromatin packing by histones due to histone deacetylation

Epigenetic regulation as a result of histone acetylation and deacetylation is seen in several diseases including cancer [39, 40]. HDAC inhibitors mediate a variety of pathways regulating epigenetic and post-translational modifications including: alteration of transcription of nuclear transcription factors including ER α , upregulation of the cell cycle gene p21, blocking the cyclin/CDK complexes leading to cell cycle arrest and differentiation, balance of the apoptotic pathway as well as affecting angiogenesis by hyperacetylation of HIF-1 α , a pro-angiogenic transcription factor and decreasing the expression of vascular endothelial growth factor receptor (VEGFR) [39]. As a result, HDAC inhibition leads to induction of cell cycle arrest, apoptosis, inhibition of angiogenesis and a reduction in cell migration and invasion. In cancer, high HDAC expression resulting in histone hypoacetylation and therefore gene repression provides a therapeutic opportunity with HDAC inhibitors.

Eighteen human HDACs have so far been identified and these are divided into 4 classes according to their homology to yeast proteins [41]. Class I, II and IV all co-ordinate zinc at the active site and are inhibited by pan-HDAC

inhibitors. Class I HDACs include HDAC1, HDAC2, HDAC3 and HDAC8 and are located within the nucleus. Class IIa HDACs include HDAC4, HDAC5, HDAC7 and HDAC9. Class IIb HDACs include HDAC6 and HDAC10 and are localised to both the nucleus and cytoplasm. Class III HDACs include Sirtuins 1-7. They do not co-ordinate Zinc but require NAD as a cofactor and are not inhibited by HDAC inhibitors [42]. Class IV HDAC inhibitors include HDAC11 only.

3.2 HDACs in Breast Cancer

HDACs that have been implicated in breast cancer include HDAC1, 2 and HDAC6. Suzuki et al have shown that a marked decrease in histone acetylation occurs following progression from normal mammary epithelium to ductal carcinoma in situ (DCIS) and DCIS to invasive ductal carcinoma [43]. The HDAC 1, HDAC2 and HDAC6 levels are also reduced but to a smaller extent. In another study, HDAC1 and HDAC3 proteins were detected in 40% and 44% of invasive breast tumours, respectively, Further HDAC1 and HDAC3 expression correlated significantly with oestrogen and progesterone receptor expression [44]. In addition, HDAC6 protein has also been detected in 65% and 77% of breast carcinomas [45, 46]. HDAC6 was also associated with ER and PR expression, with tumours expressing high levels of HDAC6 mRNA and protein were noted to have better prognosis although this was not identified as an independent factor for disease free survival (DFS) or overall survival (OS) [45]. In a similar study, overexpression of HDAC6 was not associated with improved prognosis but a subset of ER+ve patients with high HDAC6 who responded to tamoxifen had better prognosis [45, 46]. Although the expression of HDAC in tumours is associated with locally advanced, proliferating tumours, and poor patient outcomes, other conflicting studies have reported the better prognosis with HDAC expression. There seems to be a biological role in the expression of HDACs and further trials of these in reference to HDAC inhibitors are warranted [47].

3.2.1 HDAC inhibitors

HDAC inhibitors can be classified according to four classes including hydroxamates, cyclic peptides, aliphatic acids and benzamides (Summarised in Table 3.1).

Table 3-1: Classification of HDAC inhibitors.

Potency and clinical applications. Abbreviations: DLBCL, diffuse large B-cell lymphoma; NHL, Non-Hodgkin's lymphoma; AML, Acute myeloid leukaemia; CTCL, Cutaneous T cell lymphoma; PTCL, Peripheral T cell lymphoma (adapted from Lane et al, JCO Nov 2009)

CLASS OF DRUG	HDAC INHIBITION	POTENCY (<i>in vitro</i>)	CLINICAL TRIALS
Hydroxamates			
SAHA (suberoylanilidehydroxamic acid): Vorinostat, Zolinza	Pan-HDAC inhibition	μM	Phase I AML, advanced solid tumours, Phase II CTCL, DLBCL, NHL
TSA (trichostatin A)	Pan-HDAC inhibition	μM	
PXD-101 (Belinostat)	Pan-HDAC inhibition	μM	Thymoma
LAQ824	Pan-HDAC inhibition	nM	
LBH598 (Panobinostat)	Pan-HDAC inhibition	nM	
Cyclic peptides			
Depsipeptide (romidepsin, FK228)	HDAC 1 & 2 inhibitor	nM	Phase I/II CTCL, PTCL, Phase II hormone refractory prostate cancer and metastatic renal cancer
Benzamide			
MS-275 (Entinostat)	HDAC 1, 2 and 3 inhibitor	μM	Melanoma
MGCD0103 (Mocetinostat)	Class I inhibitor	nM	Phase I AML, Phase II relapsed/refractory Hodgkin's lymphoma and DLBCL
Aliphatic acids			
Valproic acid	Class I and IIa inhibitor	mM	
Sodium phenylbutyrate	Class I and IIa inhibitor	mM	

Newer hydroxamic acid derived HDAC inhibitors include givinostat (ITF2357) utilised in early phase trials of myeloproliferative disorders, proctinostat (SB939) in early trials for prostate cancer, quinosinostat (JNJ-26481588) in CTCL while abexinostat (PCI-24781) is being trialled in follicular lymphoma [48]. An HDAC6 specific inhibitor rocolinostat (ACY1215) is undergoing early clinical trials with other agents in multiple myeloma[49].

3.3 The role of epigenetics in oestrogen receptor signalling

3.3.1 Epigenetic silencing of the ER

The loss of ER expression in ER-ve breast cancer is not due to genetic alterations of the gene such as homozygous deletion, loss of heterozygosity or ER gene mutation, but rather due to changes in its epigenetic regulation. DNA methylation and histone deacetylation act in concert to regulate ER gene silencing [50]. The lack of ER expression in ER-negative breast cancers, as well as in ER-positive tumours resistant to endocrine therapy, is thought to be due in part to epigenetic changes occurring through increased deacetylation and methylation [50]. Silencing of a gene by methylation involves the generation of an inactive chromatin structure characterised by deacetylated histones. Hypermethylation results in recruitment of methyl-binding proteins that couple methylated DNA to HDAC and facilitate the silencing of genes. The CpG island of the ER gene is extensively methylated in ER-negative breast tumours [50, 51]. The hypermethylation of the CpG island may inhibit transcription by interfering with recruitment of transcription factors or transcriptional coactivators, such as AIB1, CBP/p300. In the ER+ve MCF-7

cells, the unmethylated ER promoter shows H3 and H4 acetylation and H3-K4 methylation, but little binding of methyl binding protein or DNMT (DNA methyltransferase). Conversely, in ER-ve MDA-MB-231 cells, the ER promoter is silenced by DNA hypermethylation, histone hypoacetylation, H3-K9 methylation and recruitment of methyl-binding proteins and HDAC1 [11]. In addition, methyl-transferase DNMT1 interacts physically with either HDAC 1 or HDAC 2 through its N-terminus forming a transcriptionally inactive chromatin structure [52]. Therefore, the methylation of these CpG cluster sites is associated with either reduced or absent ER expression. In normal breast tissue, DNA methylation is not observed but seen as a precursor of cancer progression [53].

Using demethylating agent 5aza-dC, Ferguson et al [54] have demonstrated the reactivation of ER transcript and functional ER protein by the ability to activate oestrogen responsive genes PR. This required treatment for 5-7 days at a dose of 0.5-0.75 μ M. Single agent treatment with HDAC inhibitor TSA tested in 3 ER-ve cell lines led to time and dose dependent re-expression of mRNA without alteration of methylation. However, the ER mRNA levels were only approximately 1-10% of levels seen in endogenous ER expressing MCF7 and T47D cells [50].

3.3.2 Oestrogen receptor positive cell lines

In ER+ve expressing cell lines, treatment with HDAC inhibitors leads to a decrease in ER expression, both at the mRNA and protein levels [55, 56]. At the transcriptional level, this has been demonstrated by treatment with HDAC inhibitors, but the concomitant treatment with TSA and cycloheximide, a protein synthesis inhibitor confirms that there is a direct role of HDAC inhibition in ER mRNA repression [55].

With regards to ER protein expression, Yi et al, demonstrated that treatment of MCF7 (ER+ve) cell lines with Vorinostat resulted in downregulation of ER protein [56]. The molecular basis for this is the hyperacetylation of Hsp90, an ER protein chaperone, by the HDAC inhibitor. Hyperacetylation of Hsp90 leads to dissociation with ER and subsequent ER ubiquitination and degradation. This mechanism is thought to be mediated by HDAC-6 specific inhibition [56]. Recently, HDAC6 has been shown to be an oestrogen-responsive gene and overexpression of HDAC6 is seen in ER-expressing breast cancers [46]. As HDAC6 is responsible for deacetylation of Hsp90 and therefore, its inhibition, it could prove a useful target in ER+ve breast cancers [57].

3.3.3 Oestrogen receptor negative cell lines.

The lack of ER α expression has been extensively investigated. The mechanism initially elucidated for ER loss was noted to be hypermethylation of the ER promoter [51]. However, only a minority of ER–ve tumours exhibit this hypermethylation [51]. ER re-expression has been characterised in ER-ve cell lines treated with demethylating agents and HDAC inhibitors by N. Davidson's group (Johns Hopkins University, Baltimore, MD, USA). Treatment of ER-ve human breast cancer cells, MDA-MB-231, with the demethylating agent, 5-aza-2-deoxycytidine (5-aza-dC) led to reactivation of expression of ER mRNA and functional ER protein. Restoration of ER function was exemplified by an increase in ERE-driven promoter activity from an exogenous plasmid, as well as expression of PR [54]. This suggests that PR may be a functional measure of ER restoration. It has also been shown that TSA (HDAC inhibitor trichostatin A) treatment of ER negative breast cancer cells, MDA-MB-231 and MDA-MB-435 can lead to a re-expression of ER-mRNA without an apparent alteration in the methylation status of the ER CpG island [58].

Combination treatment with demethylating agent and HDAC inhibitor led to ER re-expression in ER-ve cell lines. ER mRNA was induced 31-fold by treatment with 5-aza-dC in ER-negative MDA-MB-231 cells, whereas treatment with TSA had little effect. Combined treatment led to 400-fold induction of ER-transcript – which is 13 fold higher than single agent 5-aza-dC suggesting a synergistic effect [58]. Thus both agents lead to re-expression of ER, with different protein complexes associated with the ER promoter in each

case. The epigenetically reactivated ER promoter in ER negative breast cancer cells treated with both drugs achieved a chromatin profile that is similar to the ER promoter in ER positive cell lines.

Panobinostat, another HDAC inhibitor, also induced restoration of ER mRNA and ER protein without any effect on demethylation. The expression of ER mRNA was also maintained 96 hours after withdrawal of treatment [59]. Vorinostat, a pan-HDAC inhibitor, was shown to upregulate ER while downregulating EGFR in EGFR-overexpressing MDA-MB-231 cell lines. This exhibits that the effects of HDAC inhibition in ER regulation also affect growth factor receptors [60].

3.4 Response to Hormone Treatments

3.4.1 ER positive tumours

Combination of HDAC inhibitors with anti-oestrogen therapy led to an increase in both inhibition of cell proliferation and induction of apoptosis. In tamoxifen resistant cell lines overexpressing HER2+, there was a similar effect. Therefore, HDAC inhibition with anti-oestrogen therapies could provide a useful therapeutic tool in oestrogen-resistant tumours [61]. This is also similarly seen in re-sensitisation of aromatase inhibitor treatment [62].

3.4.2 ER negative tumours

As ER repression is underpinned by epigenetic mechanisms involving HDAC inhibition, the treatment of ER-ve breast cancer lines with HDAC inhibitors have shown response to anti-oestrogen treatment.

In MDA-MB-435 cell lines that are ER-ve, treatment with TSA and 5-aza-dC restored sensitivity to tamoxifen through re-expression of ER and inhibition of proliferation by tamoxifen [63]. This was similarly observed in MDA-MB-231 cells treated with panobinostat, with resulting increased sensitivity to tamoxifen, by re-expression of ER with the pan-HDAC inhibitor [59]. Distinct from the studies above, Jang et al. further observed that although tamoxifen sensitivity was seen in MDA-MB-231 cells treated with TSA, ER protein expression was not observed, despite ER transcript and PR expression[64].

In another study, MDA-MB-231 cell lines were treated with the HDAC inhibitor MS275 or etinostat and the aromatase inhibitor letrozole. Following treatment with this HDAC inhibitor, there was upregulation of ER as well as aromatase and the re-sensitisation to letrozole. In addition, there was inhibition of growth, cell migration and formation of micro-metastases when combination treatment of etinostat and letrozole was used [65]. Thus these treatments provide an effective rationale for treating ER negative breast cancer with hormone therapy in combination with HDAC inhibition.

3.5 Results

3.5.1 Characterisation of hormone receptor status in cell lines and effect of oestrogen

A number of cell lines were characterised in advance of epigenetic modulation studies. This included both ER+ve and ER-ve cell lines initially examined by western blotting. MCF7 was confirmed as ER+ve, PR+ve, as were T47D and BT474 cell lines. The MDA-MB-231 cell line was ER-ve, PR-ve. The hormone responsiveness of the cell lines was assessed with oestrogen treatment. 3×10^6 cells were grown in oestrogen-free media (phenol-red free media supplemented with charcoal-stripped foetal calf serum (FCS) for 72 hours prior to supplementation with 100nM oestrogen for 24 hours. As expected in the ER responsive MCF7 cell line, ER was downregulated and PR upregulated in the presence of oestrogen. This effect was not noted in the ER-ve, MDA-MB-231 cell line. The BT474 cell line was also oestrogen responsive. T47D cell lines have higher baseline PR compared to other cell lines but demonstrate decrease in ER and increase in PR on treatment with oestrogen (Fig 3.2).

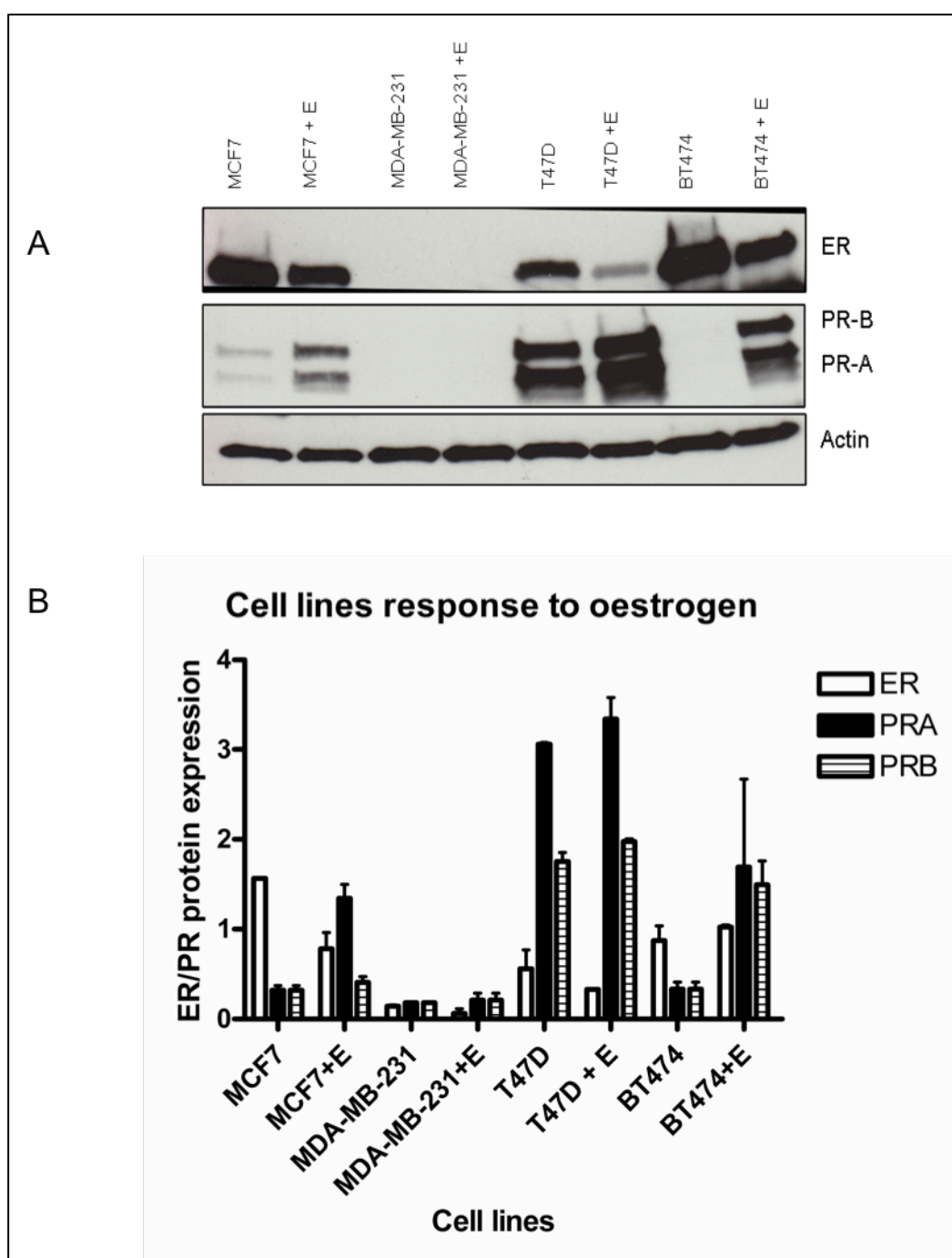


Figure 3-2: Identification of hormone receptor status in cell lines and treatment with oestrogen.

3×10^6 cells per well were grown in 6-well plates in oestrogen-free media (phenol-red free media supplemented with double charcoal stripped-FCS) for 72 hours and supplemented with 100nM oestrogen for 24hours prior to harvest. A) Western blot of cell lines. B) Densitometry of western blot. Bars represent mean densitometry (n=3) and error bars represent standard error of mean (SEM) Abbreviations: E-oestrogen, ER-oestrogen receptor, PRA (progesterone receptor A), PRB (progesterone receptor B).

3.5.2 SRB assay to determine IC₅₀ for cell lines.

SRB assays were performed to determine the IC₅₀ (the concentration at which an inhibitor reduces the response by half) of the cell lines in response to epigenetic treatments. MCF-7 and MDA-MB-231 cell lines were treated with 5-aza-dC (demethylating agent) and SAHA (HDAC inhibitor) individually at a concentration range from 0.1µM to 40µM. It can be seen from the dose response curves below that MDA-MB-231 cell lines were more sensitive to the effects of SAHA than MCF7 cell line. Despite high concentrations of 5-aza-dC, sufficient cell growth inhibition was not seen, preventing calculation of IC₅₀s of both MCF7 and MDA-MB-231 cell lines. The IC₅₀s for the cells and treatments were as follows: MCF7 treatment with SAHA, IC₅₀ 3.102 µM (IC₅₀ 2.5 µM in other publications); MDA-MB-231 treatment with SAHA, IC₅₀ 1.537 µM. (Fig 3.3)

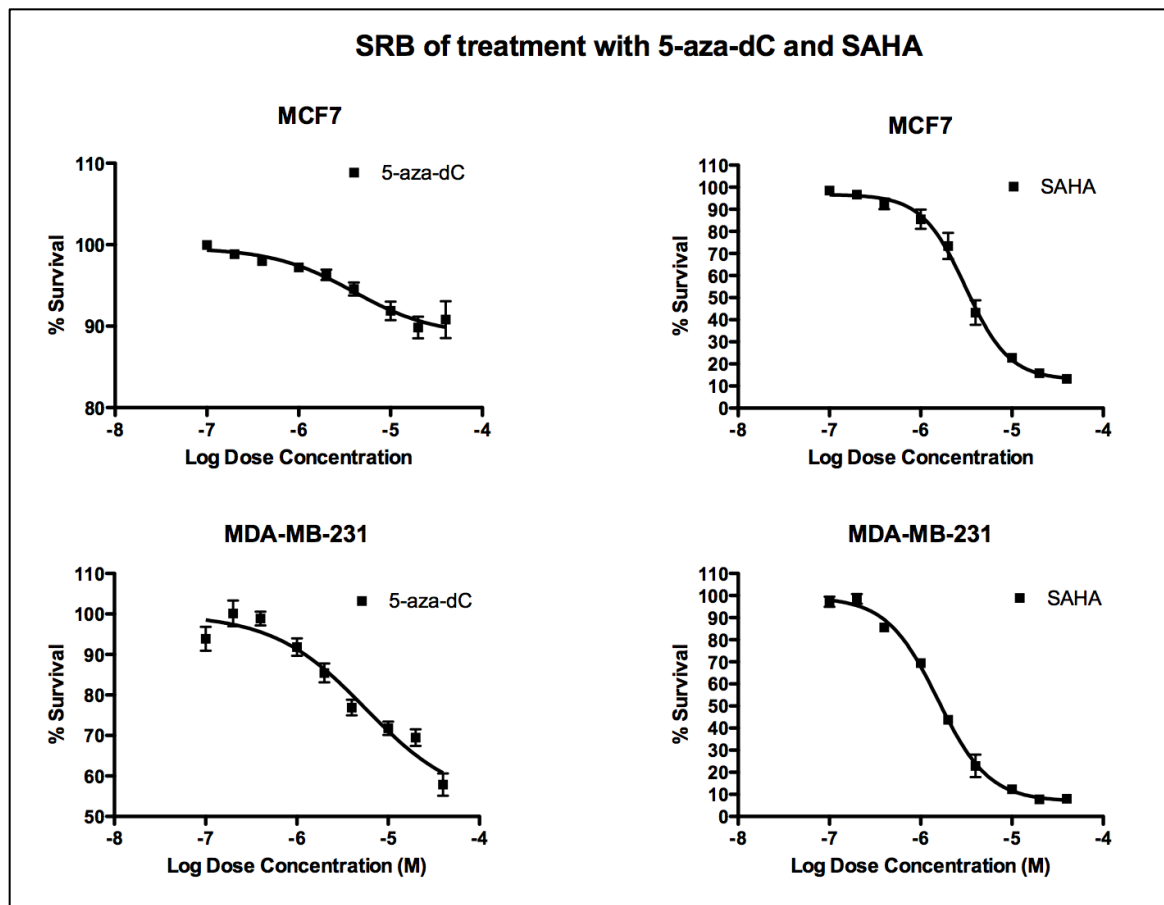


Figure 3-3: Effect of 5-aza-dC and SAHA in MCF7 and MDA-MB-231 cell growth. Cells were treated from a range of 0.1 μ M to 40 μ M. Following 72h treatment, an SRB assay was performed. Data are presented as a percentage of the control. Survival was plotted by obtaining optical density measurements from a plate reader and converted to percentages. Lines were fitted using non-regression analysis. Data presented as mean (n=3), error bars represent standard error of mean (SEM) MCF treatment with SAHA (IC₅₀ 3.102 μ M), MDA-MB-231 treatment with SAHA (IC₅₀ 1.537 μ M). Abbreviations: SAHA (Vorinostat)

3.5.3 Quantitative Real-Time polymerase chain reaction (qRT-PCR) expression of ER & PR treated with SAHA

The aim of this study was to re-express ER and therefore PR in response to epigenetic treatments in ER negative cell line. The effect of this was assessed on both ER+ve and ER-ve cell lines. To determine the effect of HDAC inhibition on the ER transcript, MCF7 and MDA-MB-231 cells were treated with vorinostat (SAHA), an HDAC inhibitor at increasing concentrations (2.5µM-10 µM) for 24 hours. Using qRT-PCR, ER and PR mRNA expression were determined. MCF7 cell lines showed a significant decrease in both ER and PR ($P<0.01$) at SAHA concentrations greater than 5µM. Conversely, the MDA-MB-231 cells showed a significant six to eight fold increase ($P<0.01$ for ER and $P<0.05$ for PR) in both ER and PR transcripts at 24 hours in a dose dependent manner (Fig 3.4).

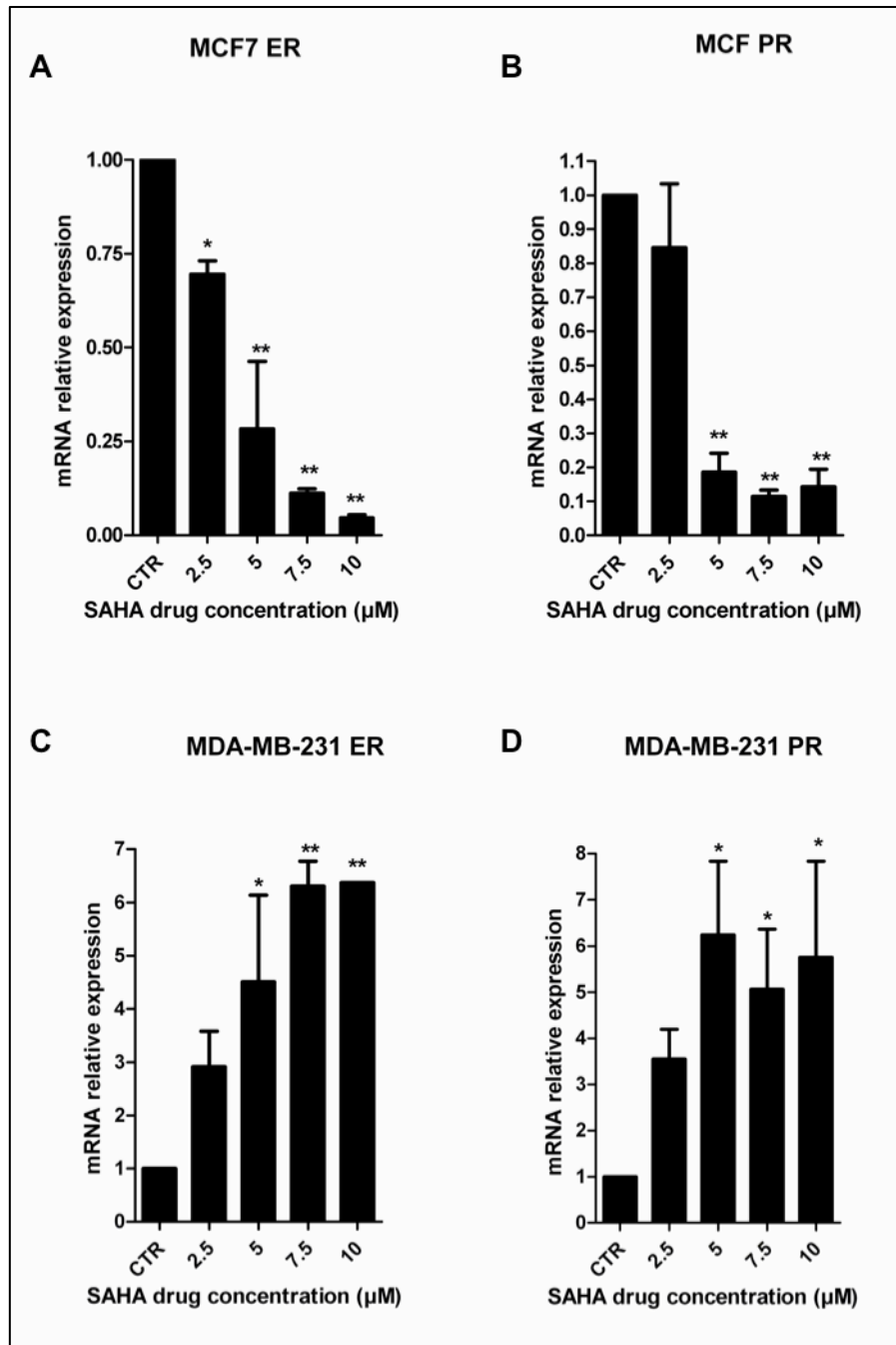


Figure 3-4: qRT-PCR of treatment with SAHA.

Relative expression of ER (A & C) and PR (B & D) mRNA, following treatment of MCF7 (A & B) and MDA-MB-231 (C & D) cells with SAHA. MCF7 and MDA-MB-231 cell lines were plated in 6 well plates at 2×10^6 cells/ml. After 24 hours, the cells were treated with a range of SAHA concentrations from 2.5 μM- 10 μM for 24 hours and then harvested for quantitative qRT-PCR. For each drug concentration, ER and PR genes were quantified relative to control conditions and corrected for expression of the control housekeeper gene (GAPDH). Mean values (n=3) and SEM are shown (*=P<0.05, **=P<0.01). Abbreviations: ER (oestrogen receptor, PR (progesterone receptor), CTR (control), SAHA (Vorinostat)

3.5.4 Combination treatment of demethylating agent and deacetylating agent

3.5.4.1 qRT-PCR

Following on from single agent treatment with an HDAC inhibitor, MCF7 and MDA-MB-231 cell lines were then treated with a combination treatment of 5-aza-dC, a demethylating agent and Trichostatin (TSA), an HDAC inhibitor to determine expression of ER and PR. The cells were treated for 96 hours in the presence of 5-aza-dC 2.5 μ M and in the last 24 hours, TSA 100ng/ml and 300ng/ml were added. This protocol was as utilised by N. Davidson's group [58]. The cells were lysed after treatment and RNA extracted. qRT-PCR analysis of the RNA showed a 150 fold ($p<0.01$) increase in ER in MDA-MB-231 cells treated with the combination treatment compared to treatment with 5-aza--dC alone, while PR expression was increased 500 fold ($p<0.01$) in MDA-MB-231 cells treated with the combination treatment. (Fig 3.5).

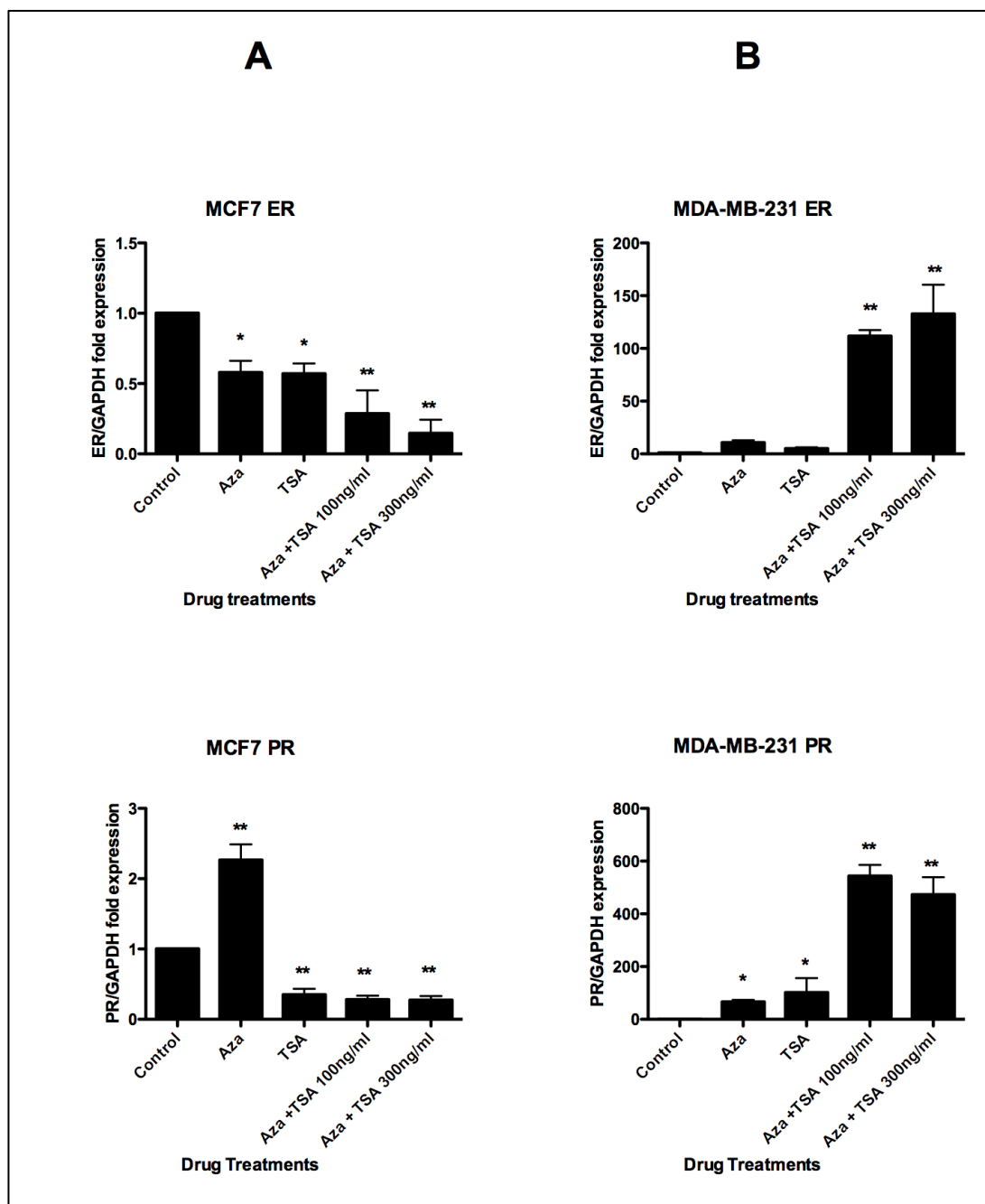


Figure 3-5: qRT-PCR following combination treatment with 5-aza-dC and TSA.

Relative expression of ER mRNA following treatment of MCF7 (A) and MDA-MB-231(B) cells with a combination of 5-aza-dC and TSA. Cells were plated in a 6 well plate and treated for 96 hours with 2.5 μ M 5-aza-dC and 100-300ng/ml of TSA for 24 hours included in the combination treatment. ER and PR genes were quantified relative to control conditions and corrected for expression of the control housekeeper gene (GAPDH). Mean values (n=3) and SE are shown (*=P<0.05, **=P<0.01). Abbreviations: ER (oestrogen receptor), PR (progesterone receptor), Aza (5-aza-dc), TSA (Trichostatin A)

The increase in ER and PR mRNA expression in MDA-MB-231 cell line relative to MCF7 cell line was examined. There was negligible increase in ER expression in MDA-MB-231 cells relative to the amount expressed by MCF7 cells. Interestingly, despite the levels of ER being negligent, the relative increase in PR was noted to be 20 fold compared with MCF7 cells (Fig 3.6)

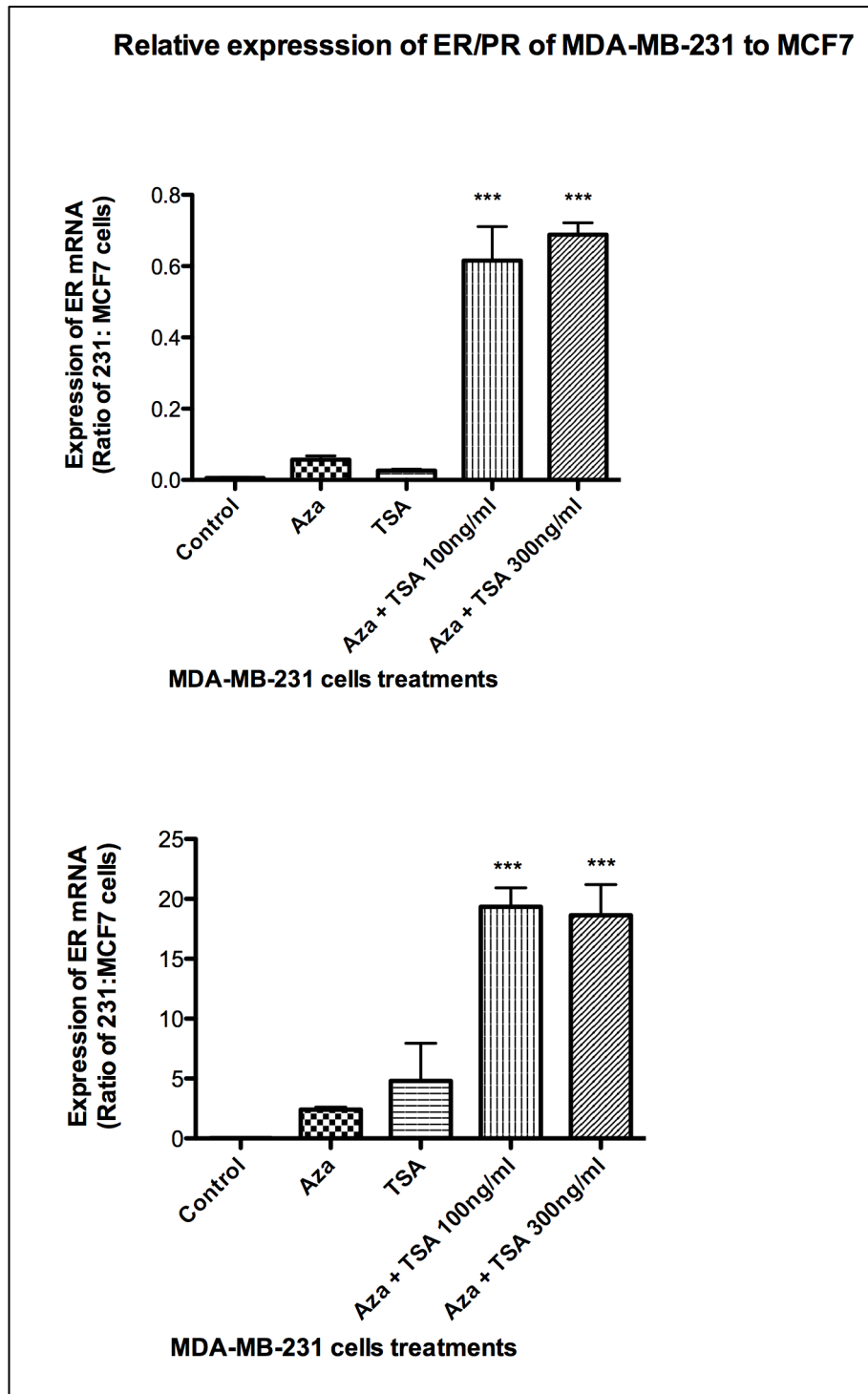


Figure 3-6: Relative expression of ER and PR mRNA in MDA-MB-231 cells relative to MCF7.

The ER and PR mRNA fold changes expressed in MDA-MB-231 are expressed relative to ER and PR mRNA expression in MCF7 control. ER re-expression in MDA-MB-231 cells is negligent for the combination treatment ($P < 0.0001$) compared to MCF7 cells. PR expression, however, is 20 fold for the combination treatment ($P < 0.0001$) relative to MCF7 cells. Abbreviations: ER (oestrogen receptor), PR (progesterone receptor), Aza (5-aza-dc), TSA (Trichostatin A). 231 (MDA-MB-231)

3.5.4.2 ER Protein expression following treatment with epigenetic modulators

MCF7 and MDA-MB-231 cell lines were evaluated for ER protein expression using demethylating agent 5-aza-dC and deacetylating agent SAHA, following on from an increase in mRNA expression with the epigenetic treatments. MCF7 and MDA-MB-231 cells were treated with a range of 5-aza-dC (1-50 μ M) and SAHA (1-10 μ M) concentrations for 24 and 72 hours. In MCF7 cells, treatment with SAHA at 10 μ M for 24 and 72 hours led to a decrease in ER protein. However, in MDA-MB-231 cells, ER protein expression was not observed with the individual treatments at the defined time points (Fig 3.7). In the combination treatment of the cells with 5-aza-dc (2.5 μ M for 96 hours) and TSA 300ng/ml for 24h, ER protein was not expressed in MDA-MB-231 cells despite increase in ER mRNA upregulation following the treatments. There was similar downregulation of ER protein in the MCF7 cells with TSA and the combination treatments. (Fig 3.7D)

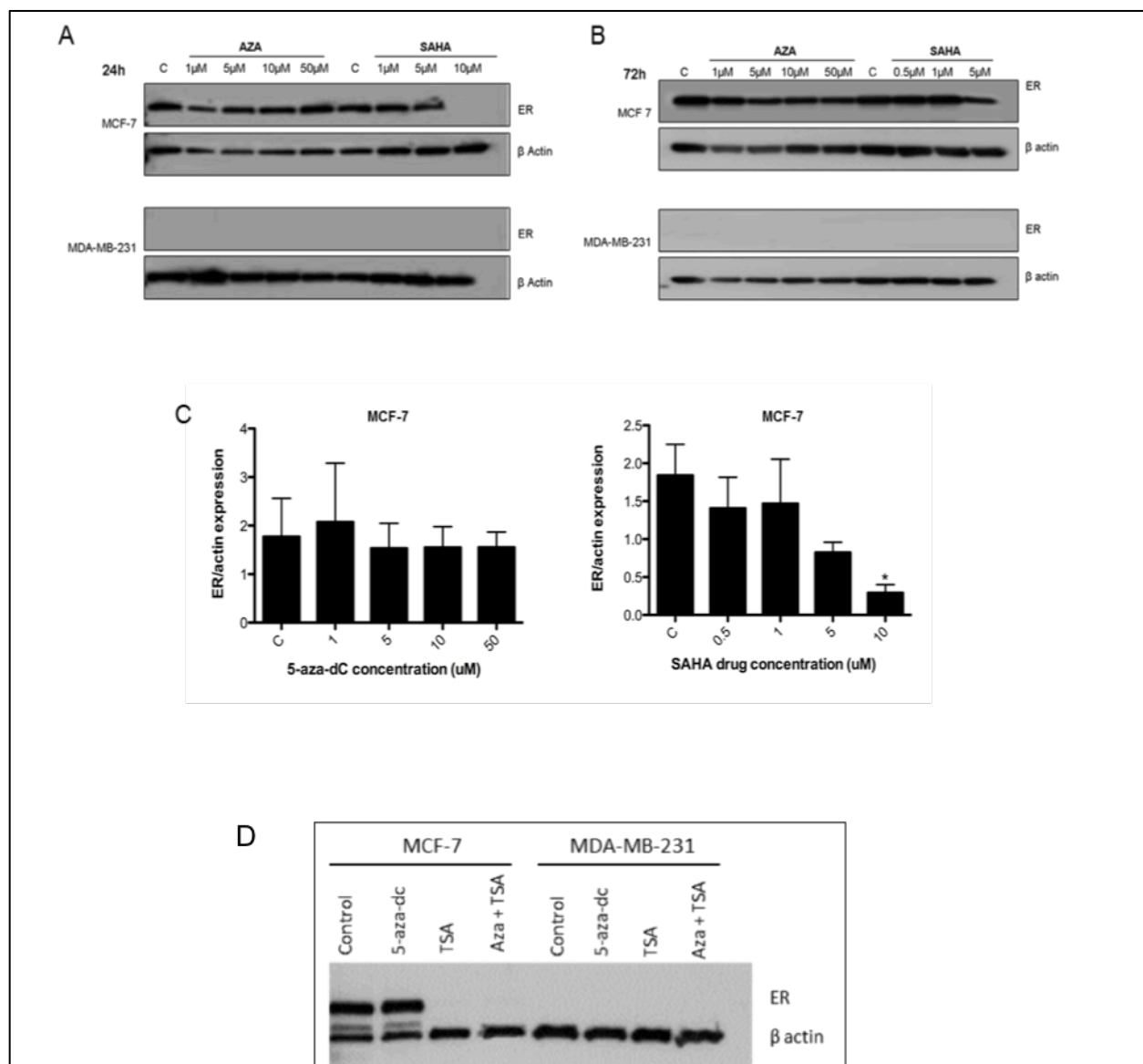


Figure 3-7: Western blot of ER protein expression.

MCF7 and MDA-MB-231 cells were plated in 6 well plates and treated with 5 aza-dC (1-50μM) and SAHA (0.5μM to 10μM) over 24(A) and 72 hours (B). Lysates were evaluated for protein using western blotting using the ER antibody NCL-L-ER6F11 (Novacastra). ER expression in MCF7 cells was normalised to actin by densitometry(C). Results obtained from the densitometry are shown as mean ± SEM. (*P<0.05) (n=3). MCF7 and MDA-MB-231 were treated with single and combination treatment of 5-aza-dC (2.5 μM for 96h) and TSA (300ng/ml for the last 24h (D). Abbreviations: Oestrogen receptor (ER), 5-aza-dc (Aza), Vorinostat (SAHA).

3.5.5 Treatment with SAHA and oestrogen to potentiate ER expression

MCF7 and MDA-MB-231 cell lines were treated with SAHA in the presence and absence of oestrogen to potentiate and increase ER expression. ER protein was determined by western blotting. Cells were grown in oestrogen-free media and subsequently treated with SAHA 2.5 μ M for 6,12 and 24h followed by oestrogen (100nM) for 24h. ER expression in MCF7 cell lines did not diminish over the time points observed and in the presence of oestrogen the ER expression was unchanged. In the MDA-MB-231 cell lines, ER re-expression was not observed by treatment with SAHA and the synergistic treatment with oestrogen expectedly did not increase ER re-expression (Fig 3.8)

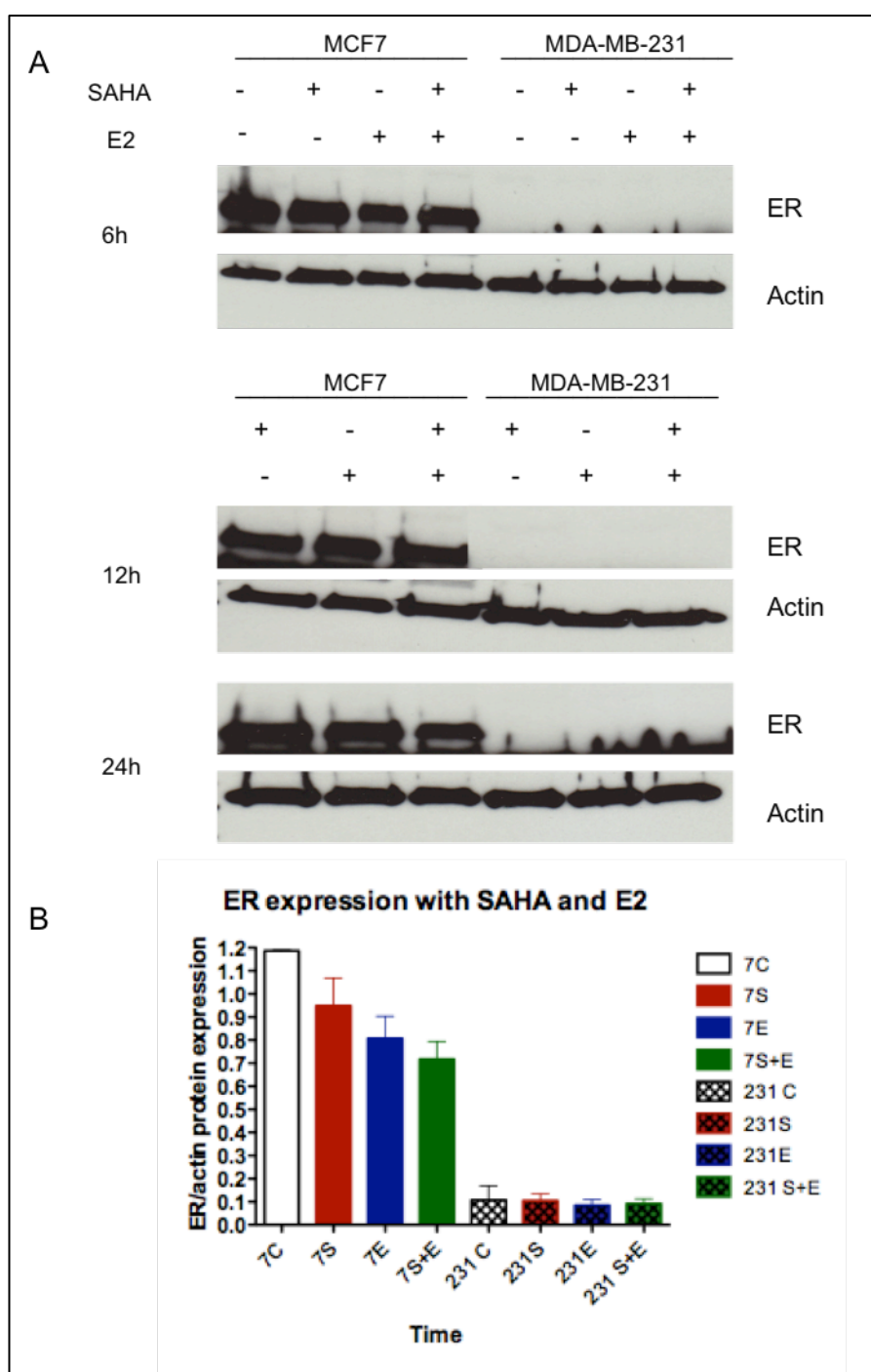
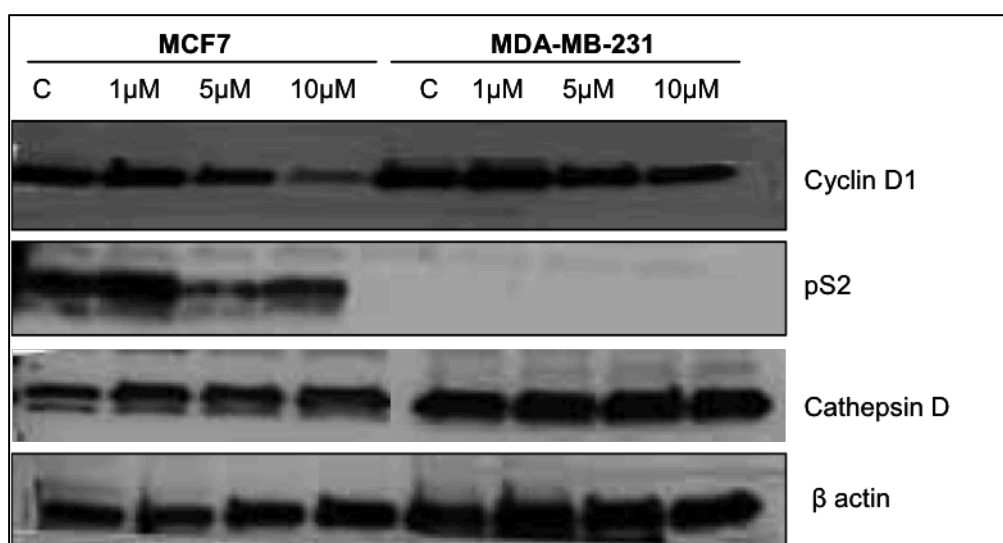


Figure 3-8: Western blot of MCF7 and MDA-MB-231 cell lines with SAHA and E2 treatment.

MCF7 and MDA-MB-231 cell lines were grown in phenol-red free, charcoal-stripped FBS media (oestrogen-deplete). Cells were subsequently treated with 2.5 μ M SAHA for 6h, 12h and 24h and followed by incubation with 100nM oestrogen for an additional 24h (A) Densitometry of western blot (B). Abbreviations: 7(MCF7), 231 (MDA-MB-231), C(control), S (SAHA), E (oestrogen), S+E (SAHA and oestrogen), ER (oestrogen receptor), SAHA (Vorinostat), E2 (oestrogen). Results obtained from the densitometry are shown as mean $\pm$ SEM (n=3).

3.5.6 Effects on oestrogen regulated genes

Although the effect on ER mRNA and protein is only observed in MCF7 cells, the HDAC inhibitors also have an effect on oestrogen regulated genes such as cyclin D1, pS2 and cathepsin D. pS2 and PR are potential markers for hormone-dependent breast cancer. Cyclin D1 is a downstream target of ER. Although Cathepsin D is known to be induced by oestrogen in ER breast cancer cell lines, it is however, also constitutively overexpressed in ER-ve tumours [66]. Cyclin D1 and pS2 protein expression were downregulated in the presence of increasing concentration of SAHA over 24 hours, reflecting the trend seen in ER and as oestrogen regulated genes in MCF7 cells (Fig 3.9). Cathepsin D expression was not diminished in the presence of SAHA in both the ER+ve and ER-ve cell lines.



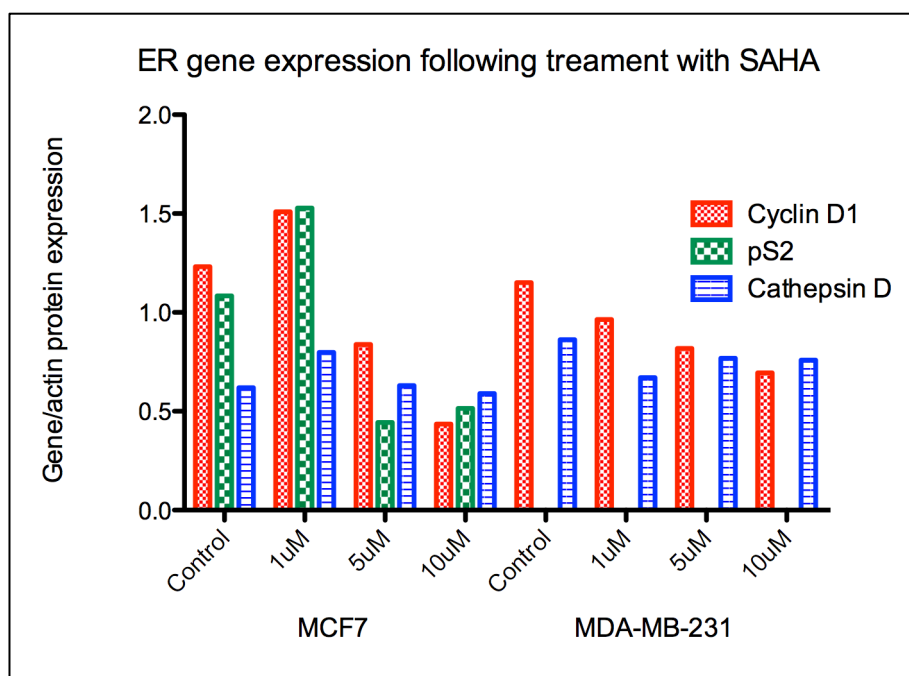


Figure 3-9: Western blot of effect of SAHA on oestrogen regulated genes. MCF7 (2×10^6 /ml) and MDA-MB-231 (3×10^6 /ml) cells were grown in 6 well plates in oestrogen-free media and further treated with increasing doses of SAHA (1-10 μ M) for 24 hours. Western blot of lysates were examined with cyclin D1, ps2 and Cathepsin D antibodies

3.5.7 ER antibody specificity

In view of the lack of ER protein expression in the treatment of MDA-MB-231 with a demethylating and a deacetylating agent, various antibodies were utilised to assess the quality and specificity of the antibody. With respect to this, a range of antibodies against different ER epitopes were examined. The NCL-L-ER6F11 ER antibody by Novacastra detects the full length of alpha form of the oestrogen receptor. The sc-543 antibody from Santa Cruz maps the C-terminus of ER α of human origin. In addition, the Santa Cruz antibody sc-8002 is specific for an epitope mapping between amino acids 570-595 at the C-terminus of ER α of human origin. ER expression was not detected in MDA-MB-231 cells with any of these antibodies. In the MCF7 cells, the

response to SAHA was maintained with all the antibodies tested (Fig 3.10). MDA-MB-231 cells from different laboratories were also tested without success (data not shown).

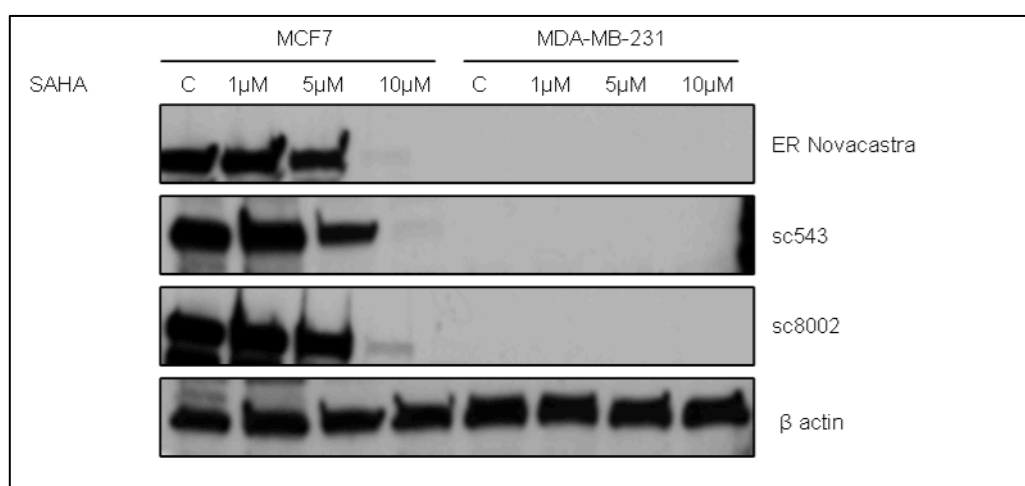


Figure 3-10: Western blotting to detect ER using a range of ER antibodies. MCF7 and MDA-MB-231 cells were treated with increasing concentrations of SAHA for 24 hours (1-10 μ M) and lysates examined for ER by western blotting with 3 different ER antibodies (ER Novacastra, sc543 and sc8002) Abbreviations ER, Oestrogen Receptor; sc – Santa Cruz antibodies.

3.6 Discussion

In breast cancer, approximately one third of cancers lack ER α . In addition, a small number of cancers that are ER+ve lose ER during tumour progression. There is increasing evidence that silencing of the ER is due to activity of transcriptional repression complexes such as DNA methyltransferase (DNMT), histone deacetylase (HDAC) or methyl CpG binding protein/HDAC [54, 67]. This therefore accounts for tumour aggressiveness and lack of response to endocrine therapy. Previous reports by the Davidson group [50, 57, 68] have demonstrated ER mRNA and protein re-expression by epigenetic manipulation of ER-ve MDA-MB-231 cells with deacetylating (HDAC inhibitors) and demethylating treatments. Further, these epigenetic treatments have also been used to resensitise ER-ve cell lines to endocrine therapy [65, 68].

In this thesis, in order to further study this effect of epigenetic treatments on ER expression, various cell lines were initially identified to represent the clinical spectrum of breast cancer. These included the MCF7, T47D and BT474 cell lines that express ER and the MDA-MB-231 cell line that does not express ER.

In my work, MCF7 and MDA-MB-231 cells were treated initially with the HDAC inhibitor SAHA to evaluate the expression of ER mRNA on these cell lines, using qRT-PCR. SAHA inhibits class I and II HDACs (HDAC1, 2, 3 and 6), as well as causing growth arrest and apoptosis at higher doses [69]. In MCF7 cells ER and PR mRNA expression was downregulated in the presence of increasing SAHA concentration at 24 hours. Yi et al have shown that the

downregulation of ER mRNA is due to direct inhibition of ER mRNA by SAHA based on co-treatment with a protease inhibitor [56]. In MDA-MB-231 cells, in my study, upregulation of ER and PR transcript (approximately 8-fold), following treatment with the deacetylating agent SAHA, was noted.

In comparison to the previous data by Yang et al [58], activation of ER was observed with synergistic treatment of DNA methyltransferase and HDAC inhibition. In the presence of 5-aza-dc or TSA alone, there was little upregulation of ER mRNA in the ER-ve cell lines, but the combination of the two treatments (5-azadC for 96 hours followed by TSA for 24 hours) resulted in a 150-fold increase in ER transcript and 500 fold increase in PR mRNA in the MDA-MB-231 cells. Relative to endogenous ER expression of MCF7 cells, the level of ER transcript expressed by MDA-MB-231 cells was 1% of that expressed by MCF7 but the level of PR transcript was relatively high at 20%.

However, this upregulation of ER mRNA in the ER-ve cell line was not reflected in protein expression in any of the treatments with SAHA, TSA or the combination treatments of 5aza-dC and SAHA and 5aza-dC and TSA. Issues pertaining to antibody selection were ruled-out following evaluation of a number of antibodies from Novacastra and Santa Cruz (used by the Davidson group), targeting a range of ER epitopes. Here, the MCF7 cell line showed a decrease in ER protein at 10 μ M SAHA but upregulation of ER in ER-ve cell lines was not observed. Yi et al suggest that the depletion of ER in ER+ve MCF7 cell lines occurs through translational and post-translational mechanisms. In addition, it has been demonstrated that the depletion of ER protein is due to degradation via the ubiquitin-proteasome pathway as well as

inactivation of chaperone heat-shock protein-90 (Hsp90), rather than direct inhibition of transcription. Hyperacetylation of the chaperone ER protein, Hsp90, by the HDAC inhibitor dissociates ER from its chaperone protein and promotes its degradation, is supported by co-treatment with a protease inhibitor in previous studies [56]. However, in the ER-ve MDA-MB-231 cell line, due to the low levels of ER and PR mRNA relative to endogenous production by MCF7 cells, it is likely that the western blot techniques could not have detected very low levels of protein produced.

PR mRNA re-expression by the demethylating and deacetylation treatments suggests that either ER mRNA re-expression eventually leads to ER protein re-expression (which is not detectable by the western blot technique) and the functional protein leads to PR mRNA expression, or that the PR promoter is also methylated and deacetylated which could account for its expression by the treatments in the ER-ve, PR-ve MDA-MB-231 cell lines. Wargon et al [70], have described PR-A silencing by methylation in constitutive-resistant tumours (tumours that have been endocrine resistant) and treatment with 5-aza-dC *in vivo* and *in vitro* led to re-expression of PR and response to anti-progestins.

To assess if the missing link relates to oestrogen-dependent activation, MCF7 and MDA-MB-231 cells were grown in oestrogen-deprived media. The cells were initially treated with SAHA 2.5 μ M for 6,12, and 24h and further treated with oestradiol 100nm for a further 24h. ER protein expression was not demonstrated in the ER-ve cell line even with the supplementation of oestradiol.

The oestrogen regulated gene pS2 is restricted to breast cancer cells and its expression is induced by oestrogen in MCF7 cell lines [71]. The cyclin D1 gene CCND1 is amplified in 20% of breast cancers while protein is over-expressed in 50% of breast cancers. Further, immunohistochemical staining for cyclin D1 is seen in oestrogen receptor positive breast cancers [72]. Overexpression of cathepsin D gene and protein is noted in most metastatic breast cancer cell lines. Cathepsin D gene expression is increased by oestrogen and growth factors in ER+ve cell lines but by an unknown mechanism in ER-ve cell lines [73]. pS2 and cyclin D1 were downregulated in MCF7 cell lines in the presence of increasing concentrations of SAHA suggesting a relevant functional response upon ER downregulation; cathepsin D was unchanged. pS2 was not expressed in MDA-MB-231 while cyclin D1 and cathepsin D were constitutively expressed in this cell line and did not change with treatment indicating a non-functional response to deacetylation as well as lack of relation to oestrogenic regulation. The finding of PR mRNA expression following epigenetic treatment is however encouraging but PR protein expression showed a similar trend to the ER protein with no expression seen.

The epigenetic treatments were also tested other of MDA-MB-231 cell lines obtained from other sources, but similar results were obtained negating the issue of quality of cell lines (data not shown).

In summary, ER expression in ER-ve MDA-MB-231 cell lines can be re-expressed at the transcript level with both SAHA and the combination of 5-aza-dc and TSA. ER protein was not expressed with epigenetic treatments in

ER-ve cell line MDA-MB-231. The most likely reason for this is that protein expression (e.g. in relation to native expression in MCF7 cells) is too low to enable detection. ER is downregulated in the ER+ve MCF7 cells at both mRNA and protein levels.

3.7 Future work

Epigenetic treatments are currently being combined with chemotherapy, molecular targeted agents and radiotherapy in the treatment of both haematological and solid tumours.

In breast cancer, endocrine resistance de novo or during tumour progression is well known. Therefore, re-sensitising the ER receptor to hormone therapy either by re-expression of the silenced ER or by targeting other growth signalling pathways may contribute to better outcomes in ER+ breast cancer.

From my work, it has been recognized that ER protein expression was not possible from epigenetic treatments of ER-ve cell lines despite re-expression of both ER and PR mRNA. It may require better optimisation of treatments including time course and dose of treatments. The addition of a proteasome inhibitor to these treatments may be helpful in understanding whether ER protein is degraded, if it is produced albeit in smaller quantities not detectable by western blotting. Additionally, it has been noted that HDAC inhibitors acetylate ER chaperone protein Hsp90, which may account for an unstable ER protein being formed and degraded, would be a further area of interest to evaluate.

Chapter 4 Pre-clinical development of a Progesterone receptor (PR) ligand for PET imaging of Breast Tumours

4.1 Progesterone receptor

In humans, progesterone is produced by the ovaries, the adrenal glands and in the placenta during pregnancy. Its effect on target tissues is mediated by the progesterone receptor. Both the oestrogen and progesterone receptor belong to the nuclear receptor superfamily [74]. The two isoforms of the progesterone receptor PR-A (94kDa) and PR-B (114kDa) specifically bind progesterone and are induced by oestrogens from a single PR gene [75] (Figure 4.1). PR-A is a truncated form of PR-B in lacking the first 164 N-terminal amino acids. The progesterone receptor structure is similar to other steroid receptors (Fig 4.1), characterised by three functional domains: a ligand binding domain (LBD) at the carboxy-terminal, involved in ligand binding and containing a transcription activation domain AF-2; DNA binding domain (DBD), responsible for receptor dimerization and binding to target DNA sequences, and an amino-terminal domain [75]. The other transcriptional activation domain AF-1, is located towards the N-terminus end of the receptor and is ligand-independent. AF-1 functions independently of ligands, in contrast to AF-2, which is ligand-dependent.

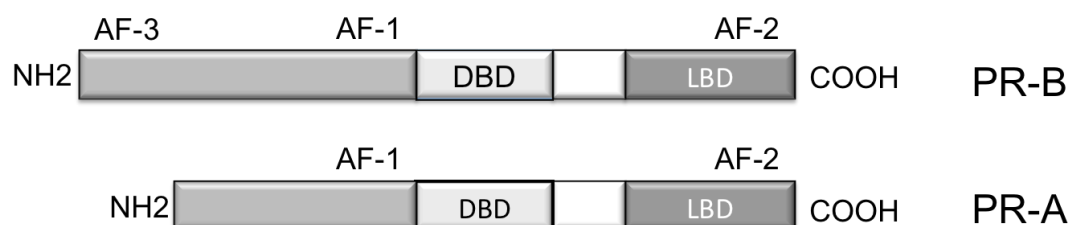


Figure 4-1: Progesterone receptor gene structure.

The structure consists of a DNA binding domain (DBD) and Activating Function 1 (AF-1), Activating Function-2 (AF-2) and Activating Function-3 (AF-3) (Adapted from Cui et al, JCO 2005)

The steroid hormones 17β -oestradiol and progesterone are necessary for the development and physiology of the normal breast [76] and have been implicated in mammary carcinogenesis. In mice, oestrogens are responsible for pubertal development, while progesterone drives cellular proliferation in the adult mammary gland [77]. This is similarly seen in post-menopausal hormone replacement therapies (HRT) where the combination of oestrogens and progesterones increase mammary proliferation over oestrogen only treatments [78].

In normal mammary breast in mouse and human, ER and PR are co-expressed in non-proliferating luminal cells that are nearby ER/PR negative proliferating cells. The hormone positive (ER/PR +ve) cells act as sensor cells in response to the steroid hormones, secrete paracrine factors RANKL, Wnt4 and amphiregulin in both species that bind to hormone receptor negative luminal or basal cells [77]. In addition, progesterone enables proliferation of hormone positive cells by a cell-intrinsic, cyclin D1 activation.

4.2 Progesterone and PR signalling in breast cancer

Progesterone has been identified as a risk for breast cancer independent of oestrogen. From a clinical perspective, studies including the Women's Health Initiative and the Million Women study [79-81] have demonstrated the increased risk of breast cancer in women taking HRT containing the progestin medroxyprogesterone acetate (MPA). Indeed women taking the combination of oestrogen and progestin had a higher risk of developing breast cancer than

those taking oestrogen only and mortality from breast cancer was increased with the combination treatment.

In mouse models of breast cancer, initial treatment of mice with the carcinogen 7,12-Dimethylbenz(a)anthracene (DMBA) followed by the PR agonist, MPA, resulted in development of mammary tumours [82]. In female Balb/c mice, treated with continuous progestin for growth, MPA induced mammary tumours that were ER/PR+ve [83]. In human breast cancer cells, wild-type BRCA1 inhibited the activity of endogenous PR and decreased proliferation. Therefore a model of breast cancer in relation to PR includes knockdown of BRCA1 with resultant increased PR activity as well as hyperproliferation in response to PR activation [84]. In dysregulated RANKL-RANK signalling, the development of progesterone dependent tumours occurs more frequently and spontaneously in a RANK transgenic mouse model as compared to wild type models. Reciprocally, treatment with a specific RANKL inhibitor reduced the incidence and progression of progesterone-induced tumours [85]. In addition, WNT signalling also increases tumourogenesis [86].

The ratios of PR-A to PR-B are noted to change from normal breast to pre-malignant conditions to malignant cancer. In the normal breast, the proportion of PR-A and PR-B are equal. In very early pre-malignant hyperplasia, these equal ratios are also maintained, however, in atypical ductal hyperplasia, DCIS and in invasive malignant tumours, PR-A predominates [87].

4.3 Progesterone receptor as a biomarker in clinical practice

The mainstay of treatment for hormone receptor positive (ER/PR+ve) breast cancers is with endocrine therapy in the form of tamoxifen and aromatase inhibitors in the adjuvant and metastatic settings. In the treatment of breast cancer, predictive and prognostic markers determine clinical outcomes. A predictive marker determines the likelihood of response to endocrine therapy in ER, PR positive or negative cancers and can determine sensitivity or resistance to treatment. A prognostic marker will anticipate clinical outcomes for patients, independent of treatment. Therefore a prognostic marker will determine which patient is likely to need treatment and a predictive marker will determine the optimal treatment. Prognostic factors derived from clinicopathological features include tumour size, lymph node metastases, histological subtype and grade as well as lymphovascular invasion. These features are integrated within the TNM (tumour, nodes, metastasis) classification and provide prognostic value [88]. In addition, ER and HER2 have established their roles as biomarkers evaluated in the assessment of breast cancer therapy. The presence of the ER biomarker provides an index for sensitivity and direct target of tumours to endocrine therapies. The American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) has recommended the routine testing of all breast cancers for ER and PR in all newly diagnosed cases of breast cancer as well as in local and distance recurrences where possible [89].

The majority of breast cancers are ER+ve and PR+ve. As PR is regulated by oestrogen, the presence of an ER-ve, PR+ve subtype is rare and represents less than 1% of all breast cancers [90]. It is reasonable to re-assess the ER status in this subset of patients in case of a false positive result. Endocrine therapy in hormone receptor positive breast cancer reduces mortality by 30%. Further, in ER+ve patients being treated with tamoxifen, the PR status did not impact on the benefit derived from endocrine therapy. However, in patients lacking ER, PR+ve patients treated with tamoxifen showed greater benefit than PR-ve patients [91]. In a review of metastatic breast cancer patients, the response to endocrine therapy was better in tumours expressing both ER and PR than in tumours that were ER+ve/PR-ve [92].

The predictive value for PR in patients undergoing aromatase inhibitor therapy showed conflicting reports. From the ATAC trial (Arimidex Tamoxifen alone or in Combination), a hypothesis-generated report suggests that the aromatase inhibitor anastrozole (arimidex) may be more effective than tamoxifen in a subset analysis of ER+ve, PR-ve tumours [93]. However, this hypothesis was not confirmed in centrally analysed material from 1856 patients. In addition, the BIG1-98 trial with 8010 patients showed better disease-free survival of letrozole over tamoxifen in ER+ve patients, but this was not affected by expression of PR [90]. These adjuvant trials support the relationship of PR expression related to endocrine therapy and prognosis of breast cancers.

4.4 PET imaging of progesterone receptors.

ER and PR are important biomarkers as predictive and prognostic markers in breast cancer and for assessing responsiveness to endocrine therapy. Since PR expression is related to ER expression, endocrine-resistant tumours would have decreased PR levels due to the signalling pathway [94]. Therefore, the expression of PR would suggest a functional ER response system and would be a more useful way of assessing ER expression and function and therefore response to endocrine therapy [95].

The development of an ER based PET radioligand, [^{18}F]FES, has been successful in imaging of ER in primary and metastatic breast tumours [96]. Further, there has been a good correlation of tumour uptake of FES and ER expression by immunohistochemistry [97]. It has also been utilised to assess response to endocrine therapy.

The rationale for developing a PR radiotracer is to assess a functional ER response system [95]. In addition, in patients receiving hormonal therapy, e.g. tamoxifen, tumour ER would be occupied by the agonists and prevent binding of radioligand [97]. It could additionally be used to image tumours that are ER-ve, PR+ve and prevent repeated biopsies to determine steroid receptor expression.

Radiolabelling of progesterone ligands (Figure 4.2) can be utilised to image PR over-expressing breast cancers. As the majority of breast cancers are ER and PR positive, this enables targeting of localized as well as metastatic tumours. Imaging of steroid receptors require the following 4 characteristics [98]:

- (i) High specific activity – as the receptors are saturable with limited uptake capacity
- (ii) High affinity for the target receptor
- (iii) Low non-specific binding to non-target and other steroid receptors
- (iv) Favourable metabolism and clearance characteristics

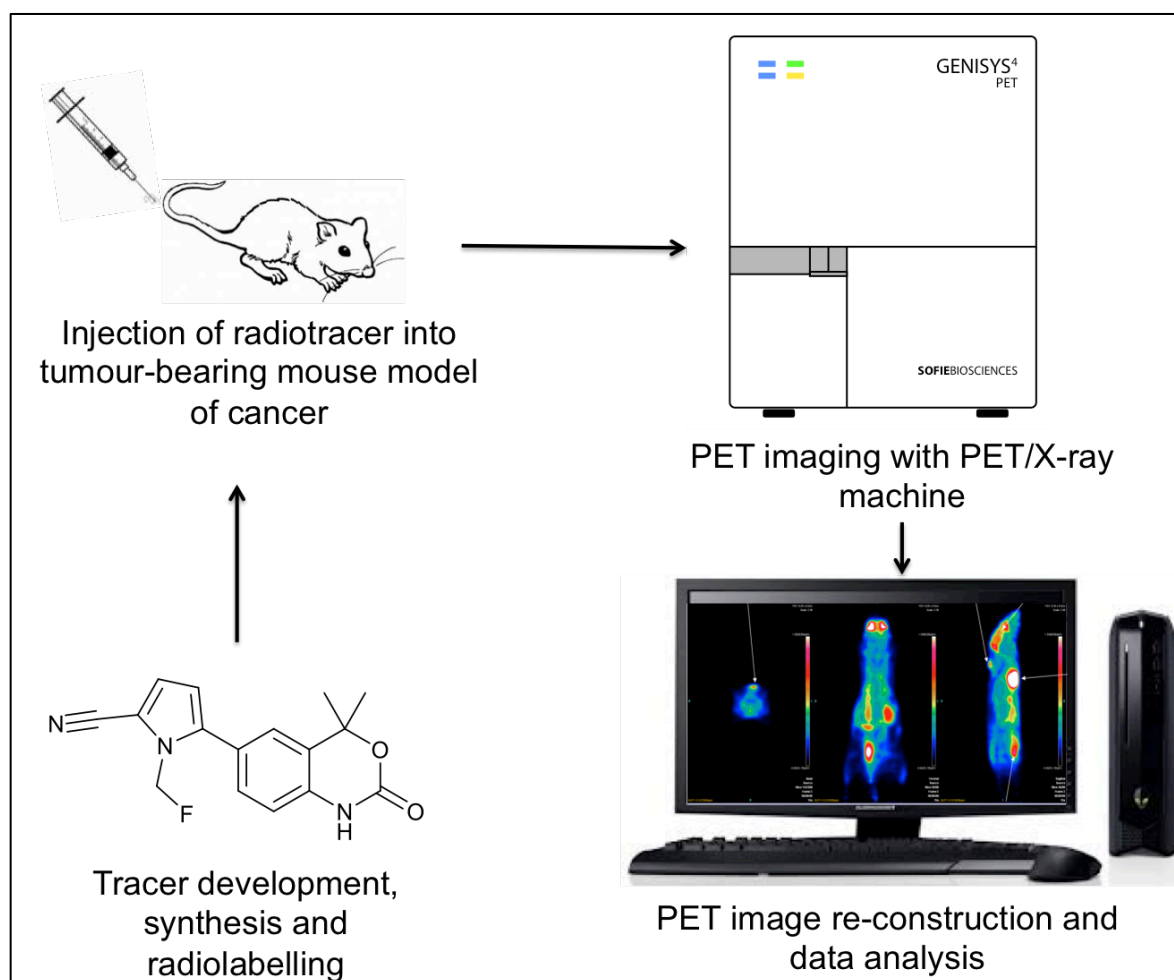


Figure 4-2: *In vivo* imaging of radiotracers.

The tracer is developed, synthesised and radiolabelled by the radiochemistry team following which it is optimised for imaging *in vivo*. Suitable xenograft models are then injected with the radiotracer prior to being imaged by the PET machine. Data from the PET machine is re-constructed by computer software prior to being analysed

Progesterone as well as synthetic progestins R5020 (promegestone) and ORG2058 have been assessed for their relative binding affinities to the progesterone receptor. In *in vitro* studies, tritiated progesterone binds to PR with a relative binding affinity of 13% compared to R5020 [99]. R5020 binds to PR with a higher affinity (775% relative to progesterone =100%)[100]. ORG2058 has a higher binding affinity to R5020 (200% relative to R5020) [100]. These findings suggest that higher binding affinities than progesterone can be achieved and may allow for higher contrast between target and non-target tissues.

21-[¹⁸F]Fluoro-16 α -ethyl-19-norprogesterone ([¹⁸F]FENP) was the first progestin to be radiolabelled. It is the fluoro-substituted analogue of the potent progestin ORG2058. [¹⁸F]FENP has a high binding affinity to the PR which is 60 times that of progesterone [101]. In *in vivo* studies, biodistribution data on rats treated with oestrogen, showed highly selective receptor uptake in the uterus. In keeping with the metabolic and excretory pathways of steroids, high hepatic and renal uptake was noted. In addition, uptake was also noted in the bone and fat uptake was associated with the lipophilic nature of the compound. As breast tissue contains a significant proportion of fatty tissue, high fat uptake would reduce the selectivity of PR in breast tumours. Studies have highlighted the avid metabolism of progesterone not only in the liver, but also in erythrocytes and mammary tumours by metabolizing enzymes such as 5 α -reductase, 3 α and 20 α -hydroxysteroid dehydrogenase and this has an effect in binding affinity to PR [102].

[¹⁸F]FENP was assessed for clinical utility in a small number of patients [95]. 8 primary breast cancer patients were imaged. 50% of PR+ve tumours were identified by imaging but did not correlate with histological findings of PR. In addition, the low target to background ratio as well as deflourination in bone and high activity in blood and normal breast precludes the use of this tracer in humans. [¹⁸F]FENP was prone to metabolism by 20 α -hydroxysteroid dehydrogenase activity and rapidly reduced to 20-dihydroprogesterin which also negates the binding of this compound to PR. Therefore, the high lipophilicity and metabolic liability are contributory to its limitations.

In an attempt to develop a more metabolically stable imaging agent to prevent deflourination and reduce lipophilicity, fluoro-furanyl norprogesterone [¹⁸F]FFNP, was developed with a high PR relative binding affinity of 190% relative to R5020 and log $P_{o/w}$ of 3.87 (cf log $P_{o/w}$ =4.66 for FENP). It was designed to be stable against deflourination, with low bone uptake. In tissue distribution studies, the compound showed PR-selective uptake in the uterus and ovaries and due to its low lipophilicity, low levels of radiotracer accumulated in bone and fat [103].

The safety and dosimetry of the progesterone analog [¹⁸F]FFNP was undertaken in addition to the feasibility of [¹⁸F]FFNP to image PR in breast cancer was assessed [104]. Twenty patients with 22 primary breast cancers underwent PET with [¹⁸F]FFNP. 16 tumours were PR+ve and 6 were PR-ve. Dosimetry studies confirmed the safety of this tracer. In the evaluation of PR imaging, patients who had not undergone prior endocrine therapy were evaluated by whole-body PET and this was correlated to tumour PR status,

assessed by *in vitro* assays. Tumour [^{18}F]FFNP uptake was assessed semi-quantitatively by maximum standardized uptake value and tumour to normal (T/N) activity ratio. There was a significant positive correlation between uptake of [^{18}F]FFNP using the T/N ratio and tumour PR status. The T/N ratio was significantly positive (2.6 ± 0.9 versus 1.5 ± 0.3 , $P=0.001$) in PR+ve cancers than in PR-ve cancers. However, the maximum standardised uptake value (SUV_{max}) of [^{18}F -FFNP] did not differ in PR+ve and PR-ve cancers. This was most likely due to patients not being on oestrogen or anti-oestrogen therapy and therefore a low level of PR. Therefore, this study demonstrates a safe and well-tolerated, non-invasive imaging modality for tumour PR evaluation in patients.

In response to endocrine therapy, in a preclinical model of breast cancer, imaging of steroid hormones with [^{18}F]FES and [^{18}F]FFNP was undertaken[105]. After baseline imaging, the mice were treated with oestradiol. The average tumour:muscle (T:M ratios) of [^{18}F]FFNP, correlated to biodistribution assay, were increased with oestradiol-treated mice in keeping with increased PR expression, which is indicative of functional ER expression. Responding and non-responding tumours were identified with imaging profiles. ER+ve, PR+ve tumours responding to the anti-oestrogen therapy, fulvestrant, demonstrated decreased [^{18}F]FES (i.e. ER), decreased [^{18}F]FFNP (i.e. PR) and decreased [^{18}F]FDG (response to therapy). In contrast, another xenograft model that was also ER+ve and PR+ve exhibited decreased [^{18}F]FES, stable [^{18}F]FFNP and stable [^{18}F]FDG. Therefore, [^{18}F]FFNP but not [^{18}F]FES, could distinguish between responders and non-responders of antioestrogen therapy in this tumour model. Therefore, imaging

of tumours with baseline [^{18}F]FES] and initial changes in [^{18}F]FFNP] could prove a useful strategy in predicting responders versus non-responders to endocrine therapy.

Steroid progestins, in addition being lipophilic and therefore allowing non-specific uptake, also show cross-reactivity with other steroid receptors, including glucocorticoid receptor (GR) and androgen receptor (AR). Tanaproget is a non-steroidal progestin with a relative binding affinity of 151% relative to R5020 while affinity for GR and AR is low. Therefore, its radiolabelled analogue, 4-[^{18}F]fluoropropyltanaproget ([^{18}F]FPTP) was assessed for PR imaging in an animal model. In these studies [106], tissue uptake of ([^{18}F]FPTP) was high in uterus and ovaries of oestrogen-primed rats. Blocking studies enabled confirmation of selectivity for PR. In view of being a non-steroidal compound, it would not be a substrate for 20-hydroxysteroid dehydrogenase, the enzyme responsible for [^{18}F]FENP failure in humans. It is noted however, that [^{18}F]FPTP does not have biodistribution features that are significantly better than [^{18}F]FENP or [^{18}F]FFNP. Nevertheless, the receptor specificity, retention time as well as being a non-steroidal compound make up for suitable characteristics to develop this radiotracer further.

In summary, the progesterone receptor ligands studies thus far have identified the limitations of utilising steroid ligands in PET imaging – these include identifying a ligand which has a high tumour to background ratio, reduced lipophilicity (fat uptake), resistance to defluorination (bone uptake) and stable metabolism. Clinical studies have also indicated the lack of correlation of

imaging with immunohistochemical data, which negates further investigation of these tracers.

Ultimately, a progesterone ligand suitable for PR imaging requires high binding affinity to PR compared with progesterone to enable contrast between target and non-target tissues. The compounds discussed in the literature have high binding affinities to PR. The limitations have included metabolic degradation of by 20-hydroxysteroid hydrogenase which can be overcome by the use of a non-steroidal progestin such as Tanaproget. The use of steroid analogues, being lipophilic, contribute to high uptake in fat tissue in the breast adding to the non-specific uptake of the compounds.

Thus far only ($[^{18}\text{F}]\text{FFNP}$) has been evaluated in patients with safe dosimetry levels. It can be utilised in evaluation of PR status in patients but cannot be used for diagnostic or staging purposes.

Currently, ER and PR status in breast tumours is determined by immunohistochemistry but molecular imaging of the steroid receptors provides a non-invasive way to assess receptor status within the tumour and at metastatic sites as well as potentially monitor response to endocrine therapy.

Due to current limitations of progesterone receptor imaging, suitable tracers for PR imaging need to be further developed.

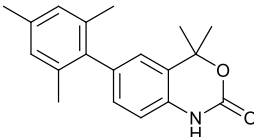
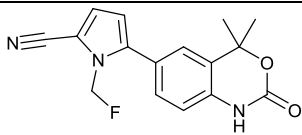
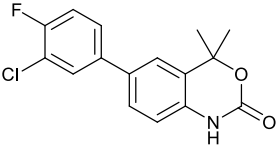
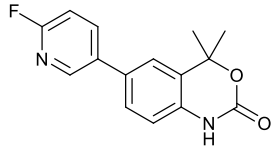
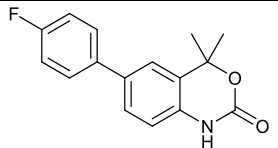
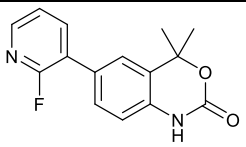
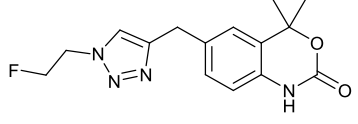
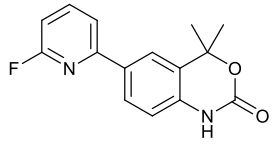
4.5 Results

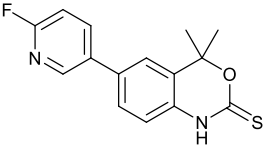
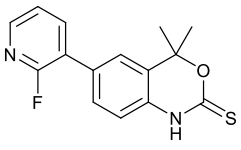
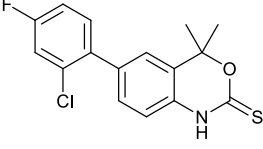
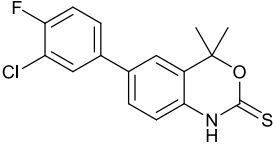
4.5.1 Progesterone analogues library for PR imaging

A small, focused library of small molecule compounds, based on the tanaproget [107] core known to bind PR with high affinity were designed and synthesised by us (CCIC) and Dr Graham Smith (University of Hull) (Table 4.1). Both carbonyl and thiocarbonyl analogues were synthesised. The carbonyl compounds included LA034, LA036, LA038, LA033, LA002, LA035, LA091, LC-01 and thiocarbonyl compounds include LA105, LA108, LA111, LA112 (Table 1). The choice of non-steroidal small molecules was predicted to increase selectivity for PR over the other nuclear receptors including ER, androgen receptor (AR) and glucocorticoid receptor (GR) when compared to steroidal-based approaches. The compounds were synthesised to probe structure-activity relationships relating to PR. The fluorine containing compounds represent a focused library of compounds that can be radiolabelled with a fluorine-18 containing prosthetic group utilising simple radiolabelling approaches.

Table 4-1: Library of putative PR-binding agents and their structure.

Compounds contain either the carbonyl or thiocarbonyl moieties.

Compound	Structure
LA034 (carbonyl)	
LA036 (carbonyl)	
LA038 (carbonyl)	
LA033 (carbonyl)	
LA002 (carbonyl)	
LA035 (carbonyl)	
LA091 (carbonyl)	
LC01 (carbonyl)	

LA105 (thiocarbonyl)	
LA108 (thiocarbonyl)	
LA111 (thiocarbonyl)	
LA112 (thiocarbonyl)	

4.5.2 The progesterone receptor activation assay

The activity of the progesterone analogues on PR was assessed using the T47D activation assay (Figure 4.3).

The carbonyl compound (LA036) and the thiocarbonyl compounds (LA105, LA108, LA111, LA112) exhibited activation of PR in this assay. LA036 activated PR in this assay with an EC_{50} of 9.4nM while thiocarbonyl compounds exhibited PR activity at the following EC_{50} : LA105, >49.6nM; LA108, 7.47nM; LA111 7.75nM, LA112 >366nM.

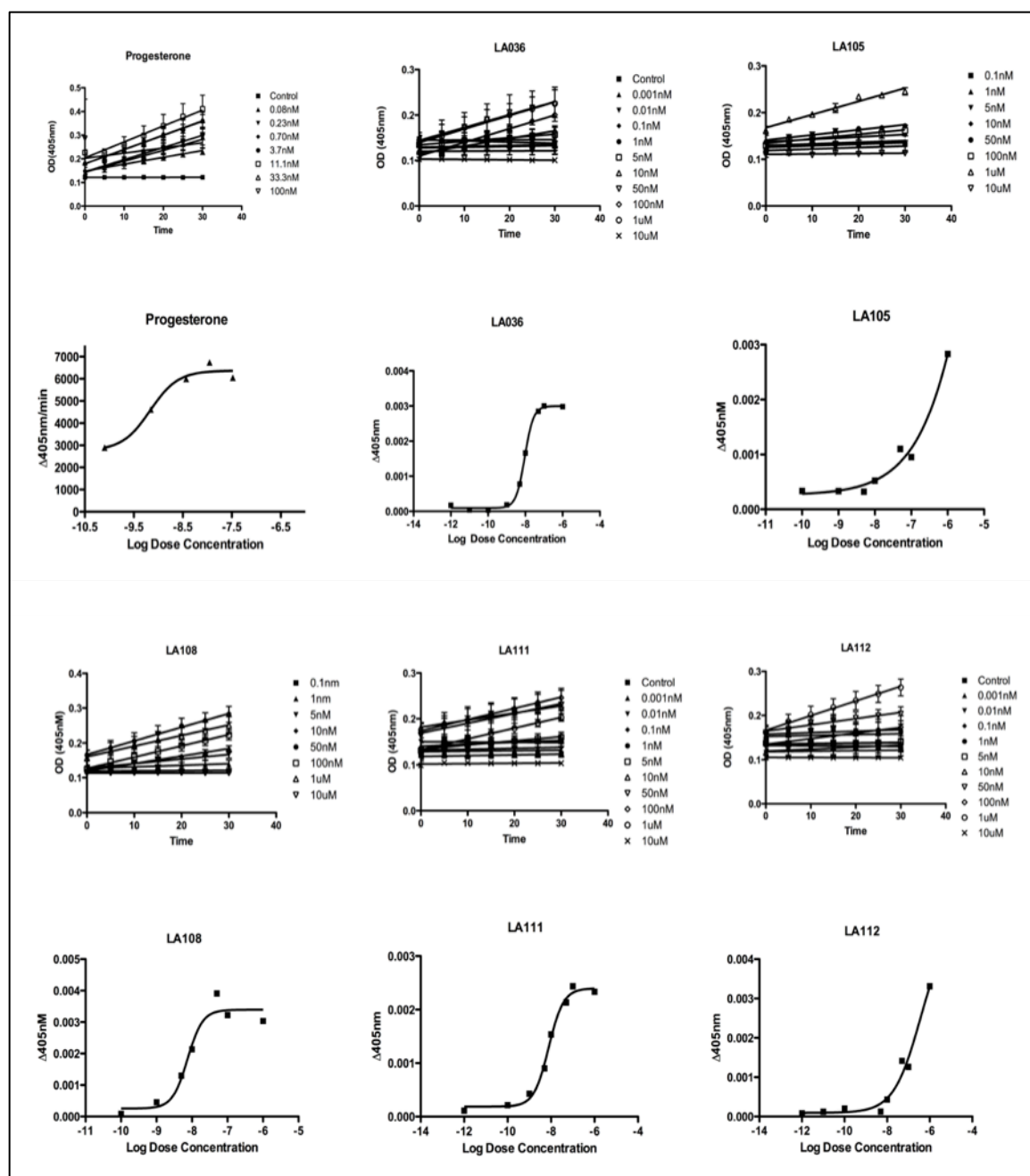


Figure 4-3: Effects of progesterone and PR analogues on alkaline phosphatase activity in T47D cells.

T47D cells were plated at 50,000 cells per well in 96 well plates in DMEM with 10% FCS. The following day the media was replaced by DMEM without phenol-red. 24h later, the test compounds were added to the media. The cells were cultured for another 24h and tested for alkaline phosphatase by the addition of p-nitrophenyl phosphate. Optical density measurements were taken at 5 minute intervals for 30 minutes at a wavelength of 405nm. The results were plotted for each of the concentrations and the change in optical density over time resulted in the calculation of the EC_{50} .

4.5.3 Progesterone receptor competition assay

The cell-free fluorescence polarization assay was used to determine the IC_{50} and therefore the relative binding affinity of the compounds that exhibited activity in the T47D activation assay.

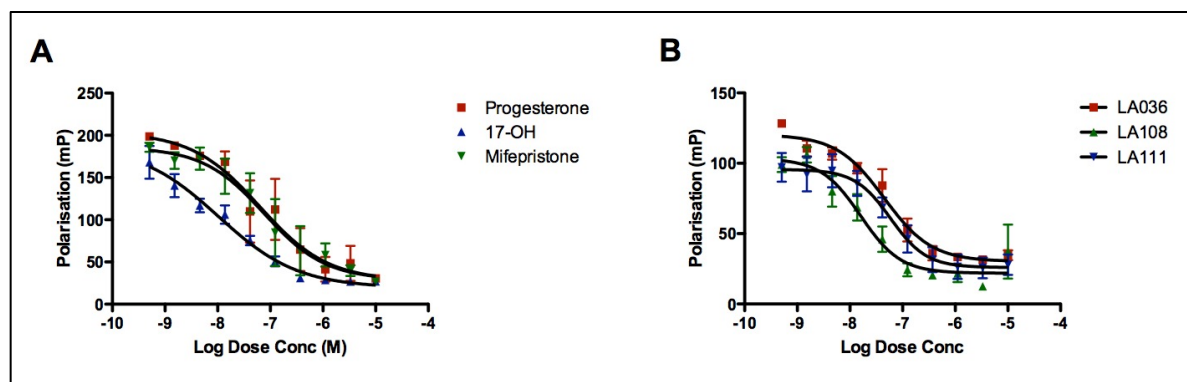


Figure 4-4: Determination of the IC_{50} and relative binding affinities of the progesterone analogues using a fluorescence polarisation assay.

Progesterone, Mifepristone (antagonist) and 17-hydroxyprogesterone (agonist) were used as controls and IC_{50} s determined (A). LA036, LA108 and LA111) were tested at increasing concentrations and IC_{50} obtained (B). $n=3$. Abbreviations: 17-OH (17 hydroxyprogesterone)

Increasing concentrations of the analogues (Progesterone, 17-hydroxyprogesterone, Mifepristone, LA036, LA108, LA111) binding to the PR-LBD, resulted in a low polarisation value. The concentration of the test compound resulting in half-maximum shift in polarisation was equivalent to the IC_{50} of the compound (Figure 4.4). The relative binding affinities of the compounds were determined by comparing the IC_{50} of the compounds in relation to the IC_{50} for the control, progesterone (Table 4.2).

Table 4-2: IC₅₀ and relative binding affinities (RBA) of progesterone analogues

Compound	IC ₅₀	RBA
Progesterone	62.85nM	n/a
17-hydroxyprogesterone	10.36nM	600%
Mifepristone	76.17nM	81.9%
LA036 (carbonyl)	44.71nM	140%
LA108 (thiocarbonyl)	15.99nM	393%
LA111 (thiocarbonyl)	59.13nM	106.3%

The carbonyl compound LA036 exhibited the most favourable profile relative to progesterone and due to radiolabelling techniques available in our laboratory, was selected for further *in vitro* and *in vivo* testing.

4.5.4 Cross-reactivity assays.

LA036 is a non-steroidal analogue of tanaproget and should not exhibit any cross-reactivity to other steroid receptors such as the oestrogen receptor (ER), androgen receptor (AR) and glucocorticoid receptor (GR), which would improve specificity of the tracer to PR and reduce non-specific uptake.

This was tested with

- (i) A549 cells

A549 cells are non-small cell lung cancer cells that exhibit high GR and do not express other steroid receptors. A549 cells were seeded in 96 well plates (25,000 cells/well) with double stripped serum in phenol red free media. 24 hours later, the cells were transfected with mouse mammary tumour virus reporter (the long term repeat contains a GR luciferase response element). Cells were treated with dexamethasone, MPA (medroxyprogesterone acetate) and LA036 at increasing dose concentrations. Dexamethasone, being a ligand for GR exhibited high luciferase activity in keeping with activation of GR reporter. MPA had minimal GR activity while LA036 showed negligent activation of GR (Figure 4.5).

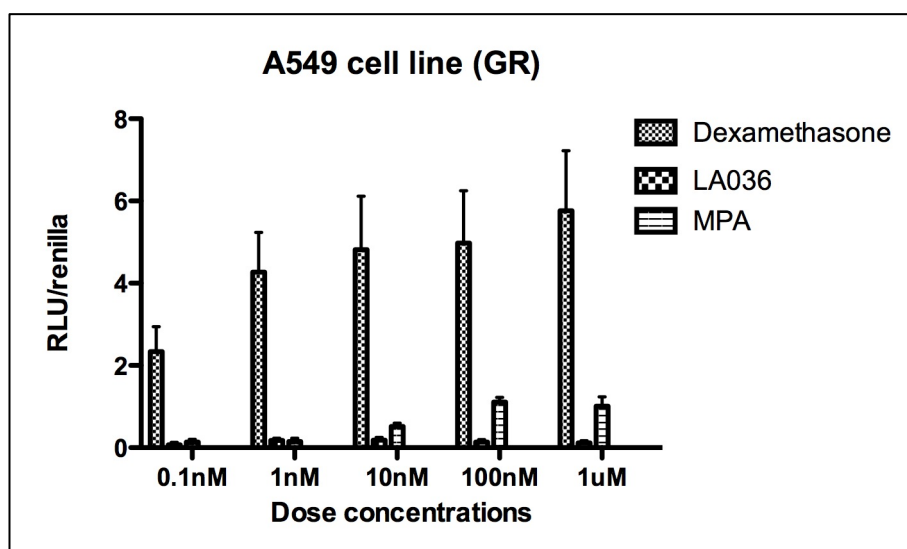


Figure 4-5: Activity of LA036 on Glucocorticoid receptor.

A549 cells (25,000 cells per well) are treated with Dexamethasone, LA036 and MPA at increasing dose concentrations after transfection with MMTV. Luciferase activity was normalised to renilla (control reporter) with the Dual-Glo Luciferase assay (n=3). Abbreviations: GR (glucocorticoid receptor), MPA (Medroxyprogesterone acetate), RLU (relative luciferase units)

(ii) LnCap cells (with AR luciferase)

LnCap cell lines that have been stably transfected with AR luciferase (kindly provided by Professor Bevan group) were treated with increasing doses of dihydroxytestosterone (DHT), MPA and LA036. Cells were seeded at 25,000 cells/well, in double charcoal stripped FCS in phenol red free media and treated after 24 hours with compounds. DHT activates the androgen receptor with up to 20-fold activation in concentrations up to 1µM. MPA, a potent progesterone, increased AR activation 5 fold with 1µM concentrations, while LA036 resulted in a 3 fold activation of GR at the same concentration (Figure 4.6).

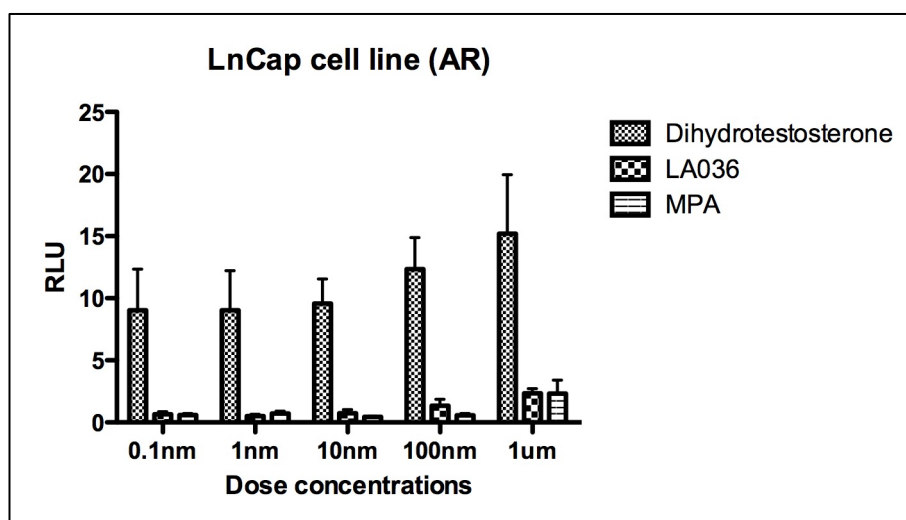


Figure 4-6: Activity of LA036 on the Androgen receptor (AR).

LnCap (25,000 cells/well) were treated with DHT (dihydroxytestosterone), MPA and LA036 at increasing dose concentrations. Luciferase activity was detected by the One-Glo assay and normalised to protein content with BCA. Abbreviations: AR; androgen receptor, MPA; medroxyprogesterone acetate (MPA), RLU; relative light units.

(iii) MELN cell line (with ER luciferase)

The MELN cell line (kindly donated by Dr Madryn Lake, CCIC, Imperial College London) is an MCF7 cells line that has been stably transfected with an oestrogen responsive promoter, which drives the transcription of the firefly luciferase transporter gene. MELN cells were grown in oestrogen-free media for 72 hours, and then seeded at 10,000 cells per well. 24h after seeding, cells were treated with oestradiol, MPA and LA036. Quantification of luciferase activity was determined by addition of D-Luciferin and activity measured by a luminescence counter. The luminescence data were normalized to the protein content of the cells. LA036 showed similar activation of the ER receptor to MPA, but not to the extent seen by oestrogen itself. Therefore, some cross-reactivity to ER is seen (Figure 4.7).

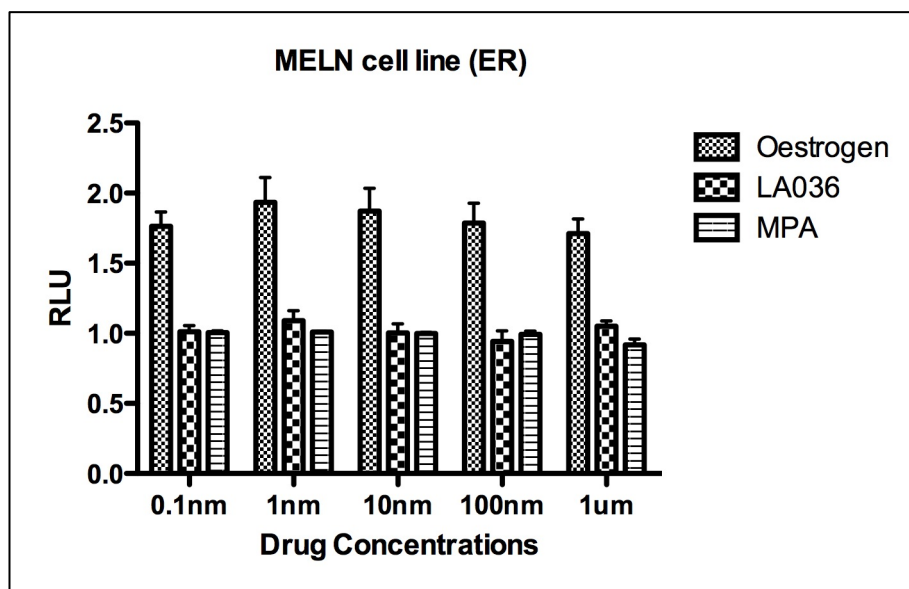


Figure 4-7: Activity of LA036 on MELN cells.

MELN cells (10,000 cells per well) were initially grown in oestrogen free media and then seeded. Following this, cells were treated with increasing doses of oestrogen, MPA and LA036 and luminescence activity determined by addition of D-luciferin and normalized to protein concentration. RLU (relative light units)

In summary, non-steroidal small molecule analogues of progesterone show less cross-reactivity than steroidal analogues with the corresponding steroidal receptors.

4.5.5 Radioactivity cell uptake assays.

MCF7, MDA-MB-231 and T47D cells were treated in the absence and presence of oestrogen for the PR radioligand, [^{18}F]LA036 in a cell uptake assay. T47D cells express higher levels of PR compared to MCF7 cells and this was reflected in the cell uptake assay. MDA-MB-231 cells also showed uptake of the radiotracer suggesting some non-specific activity. The cells were initially seeded in normal cell media (i.e. not oestrogen depleted) and supplemented with 10nM oestradiol to assess the effect on PR radioligand binding in the oestrogen treated cells. The results of the experiments are shown below (Figure 4.8)

In order to further assess the role of oestrogen stimulation on PR expression, all cell lines were grown in the presence of oestrogen-depleted media (phenol-red free media with charcoal double-stripped FCS) for 48 hours. Following this, cells were seeded in a 6-well plate for 24 hours before being supplemented with 10mM of oestrogen. Although no change in uptake was noted in relation to oestrogen supplementation, T47D cells that are high PR expressing demonstrated increased uptake compared to MCF7 cells. Non-specific uptake was again noted in PR-ve MDA-MB-231 cell lines. Although PR protein is increased in MCF7 cells in the absence and presence of oestrogen, this is not reflected with increased PR uptake in the assay. T47D cells exhibit increased PR expression with oestrogen and this is also seen in the cell uptake assay. It is possible that the levels of PR in MCF7 cells are not sufficient to detect an uptake of the radioligand.

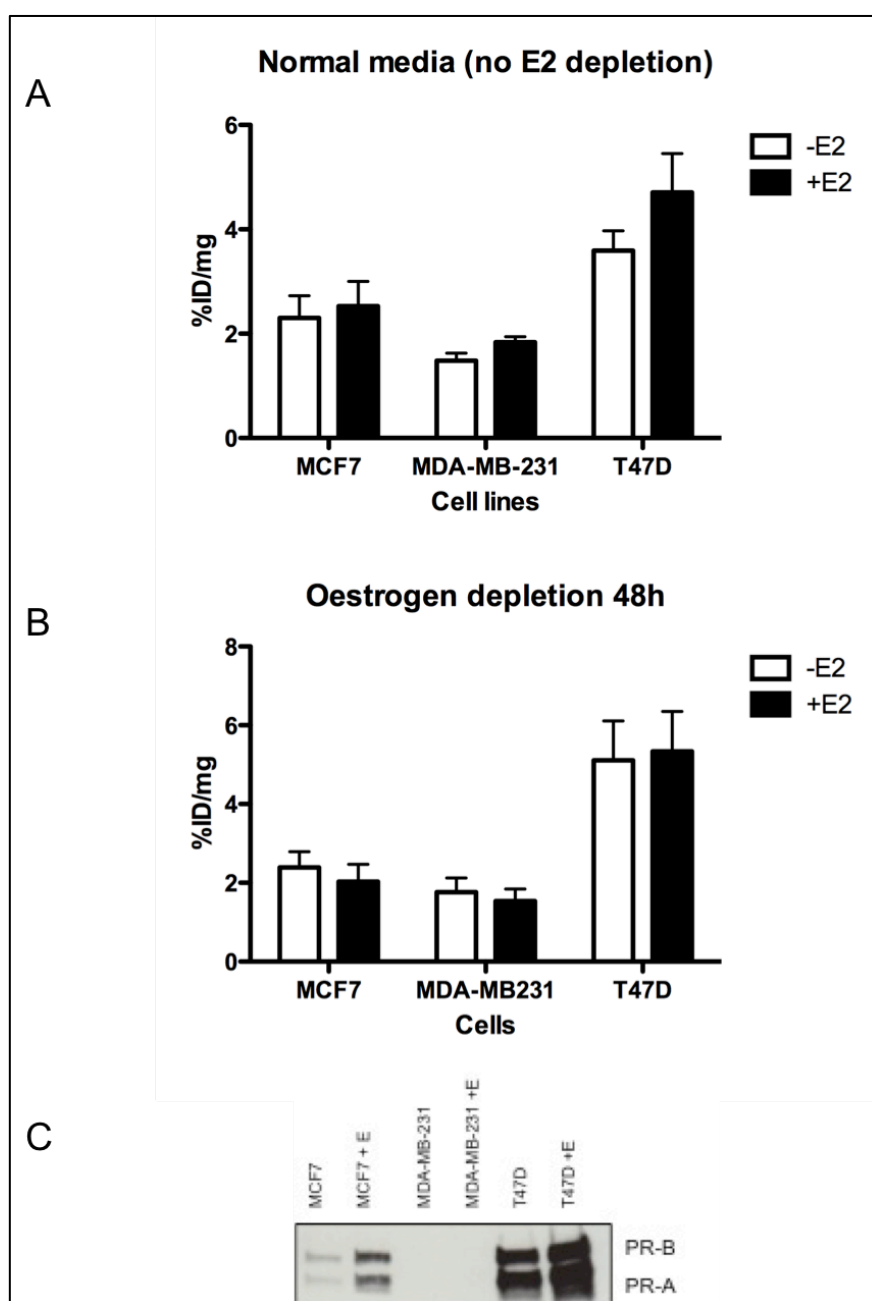


Figure 4-8: Cell uptake assay of [^{18}F]LA036 in the presence and absence of oestrogen.

Radiolabelled LA036 uptake is dependant on PR expression independent of oestrogen supplementation. MCF7, MDA-MB-231 and T47D cells were grown in normal media (A) and oestrogen depleted media (B) for 48hr (phenol-red free and charcoal double-stripped media) prior to seeding and treated with 10mM oestrogen for 24 hours. After a further 24h, 0.74MBq of [^{18}F]LA036 was added to each of the wells and incubated for 60 minutes. Activity of lysed cells was measured using a gamma counter. Correlation of PR expression is seen in the western blot experiment (C). Experiments are n=3, bars represent means and error bars represent SEM.

4.5.6 Determining specificity of [^{18}F]LA036 by blocking studies.

In order to determine the specificity of radiolabelled [^{18}F]LA036 to PR in relation to progesterone, a blocking study was conducted. T47D cells were seeded at 250,000 cells/ml in a 6 well plate. To each well containing 1 ml of media, 50 μl of progesterone was added just prior to 50 μl of [^{18}F]LA036. The specific activity of [^{18}F]LA036 was 10GBq/ μmol . Concentrations of 0-10 μM progesterone were added to the cells incubated with [^{18}F]LA036. Increased progesterone concentration decreased the binding of [^{18}F]LA036 to PR in T47D cells (Figure 4.9).

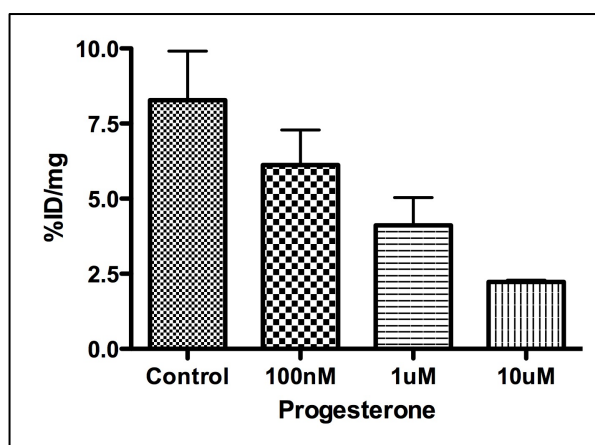


Figure 4-9: Blocking studies with progesterone.

T47D cells were treated with progesterone and [^{18}F]LA036 to determine the specificity of [^{18}F]LA036 to PR. Concentrations of 0-10 μM progesterone were assessed. The specific activity of [^{18}F]LA036 was 10GBq/ μmol

4.5.7 Cell uptake conditions (60 min washouts, timed washouts and ABC transporters)

The cell lines, MCF7, MDA-MB-231, BT474 and T47D cells lines were treated with radiolabelled progesterone receptor tracer [^{18}F]LA036 in 3 different conditions.

a) Uptake of PR tracer in the presence and absence of oestrogen and washout at 60min

Cells were treated with [^{18}F]LA036 in media for 60 minutes followed by replacement with normal media (without radioactivity) for a further 60 minutes. There was no difference noted in the 60 minute uptake between oestrogen and oestrogen-free conditions (Figure 4.10). However, replacement with radioactivity-free media resulted in complete washout of tracer with minimal activity noted in both oestrogen and oestrogen-free conditions suggesting weak interaction of the radioligand with PR.

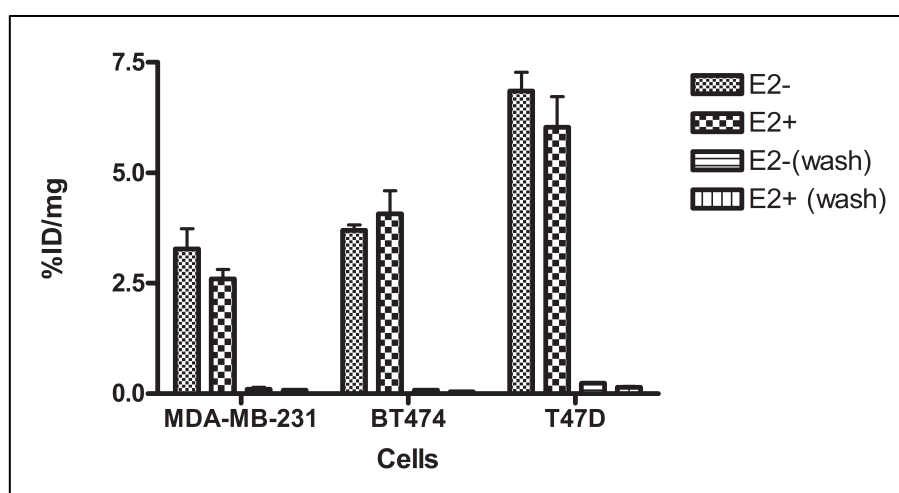


Figure 4-10: Cell uptake of [^{18}F]LA036.

Cells were treated in oestrogen depleted and oestrogen rich conditions followed by a washout of medium at 60 minutes. Cells were plated in 6 well plates as previously described in oestrogen-free media followed by supplementation of oestrogen for 24 hours prior to cell uptake assay with [^{18}F]LA036. After addition of radiotracer and incubation for 60 minutes, cell uptake was measured utilizing a gamma counter. Cells were subsequently washed and replaced with radioactivity-free for 60 minutes following which activity was measured using the gamma counter. Experiments n=3. Abbreviations. E2+ - addition of oestradiol. E2- : oestradiol not supplemented.

b) Cell uptake assay to assess time of radiotracer washout

Time points of 5, 15, 30, 45 and 60 minutes (Figure 4.11) were used to determine the exact timing of washout of the PR radiotracer [^{18}F]LA036 after earlier determination of complete washout of radiotracer at 60min. Cells were again incubated in [^{18}F]LA036 in media for 60 minutes after which the media containing radioactivity was replaced radioactivity-free media and cell uptake measured at the various time points. Uptake was high prior to the washout step. However, minimal radioactivity was again noted following washout; this was noted at all time points as early as 5 minutes of radiotracer washout.

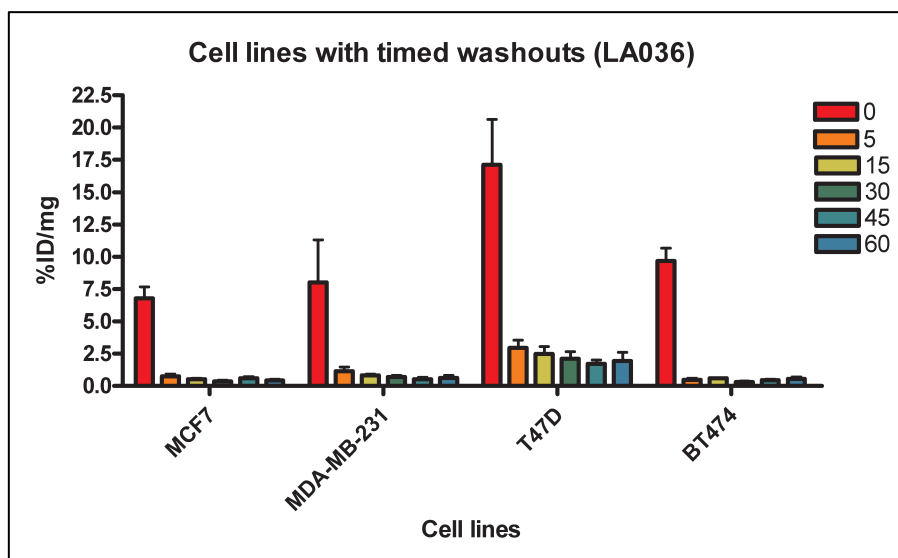


Figure 4-11: Determination of retention of [^{18}F]LA036 at time points.

Cell lines MCF7, T47D, BT474 (PR+ve cell lines) and MDA-MB-231 (PR-ve) were treated with radiotracer [^{18}F]LA036 for 60min after which media containing tracer was replaced with normal media as previously described. Radioactive uptake was determined at 5,15,30,45 and 60 minutes following washout of radiotracer. Experiments n=3.

c) Relation of cell uptake to inhibitors of ABC transporters

To further investigate the rapid washout of the radiotracer, cells were treated with a number of ABC transporter inhibitors (Zosuquidar, MK571 and FTC) to assess the mode of elimination (Figure 4.12). A time point of 10 minutes was preferred for evaluation in view of rapid washout of radiotracer. In addition, BSO, an inhibitor gamma-glutamylcysteine synthetase, lowering cellular glutathione levels, in combination with mifepristone was used in the treatment of cells to ascertain the mechanism of rapid transit of radioligand. None of these mechanisms could account for the lack of retention of the tracer in these cellular models.

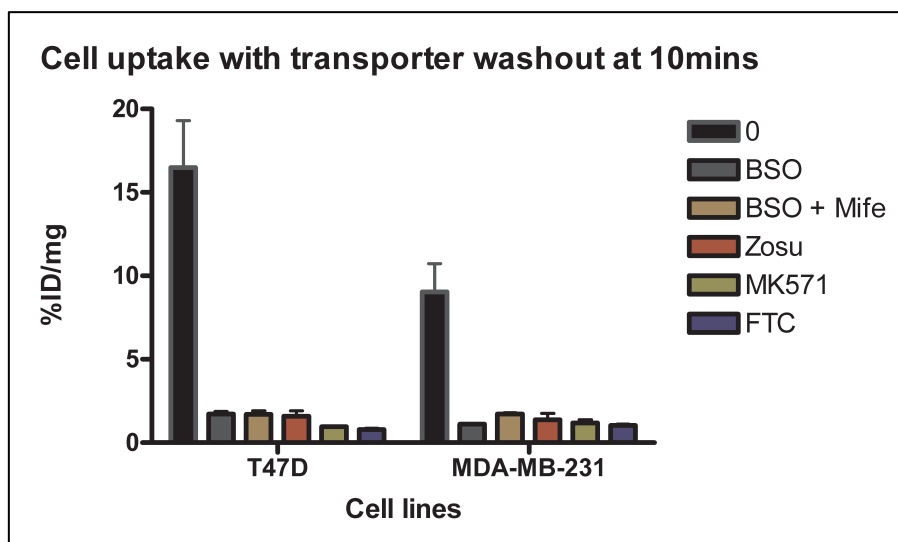


Figure 4-12: Evaluation of mechanism of rapid transit of [^{18}F]LA036.

PR+ve cell line T47D and PR-ve cell line MDA-MB-231 were treated with BSO 24hrs prior to addition of radiotracer; BSO and Mifepristone 24h prior and Zosuquidar, MK71 and FTC 1 hour prior to addition of radiotracer [^{18}F]LA036. 60 mins after addition of radiotracer, media containing compounds and radiotracer were replaced with radioactivity free media in the cells. Radioactivity was measured at 10mins to measure retention of radioligand within the cell lines.

4.5.8 In vivo uptake of LA036 (non-tumour bearing mice)

The metabolism and excretion of [^{18}F]LA036 was assessed initially in non-tumour bearing mice (Figure 4.13). LA036 uptake was assessed by PET both dynamically up to 60min, as well as static PET scans at 50-60min. Uptake was noted in the bone (defluorination) as well as high uptake in the liver, gallbladder and small intestine in keeping with metabolism of the tracer. Biodistribution studies also reflected similar uptake in the bone, liver and small intestine as well as excretion through the kidney and bladder.

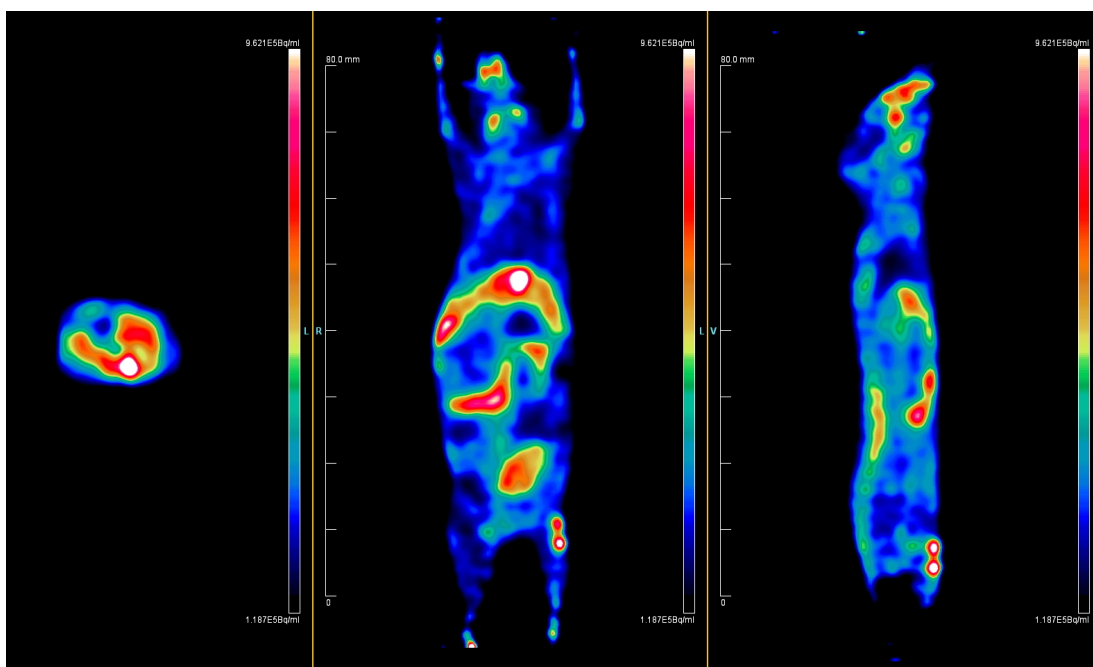


Figure 4-13: Assessment of [^{18}F]LA036 in non-tumour bearing mice.

Dynamic and static (50-60min) PET scans were done to assess the uptake of LA036 in the various organs. This was correlated to the biodistribution data.

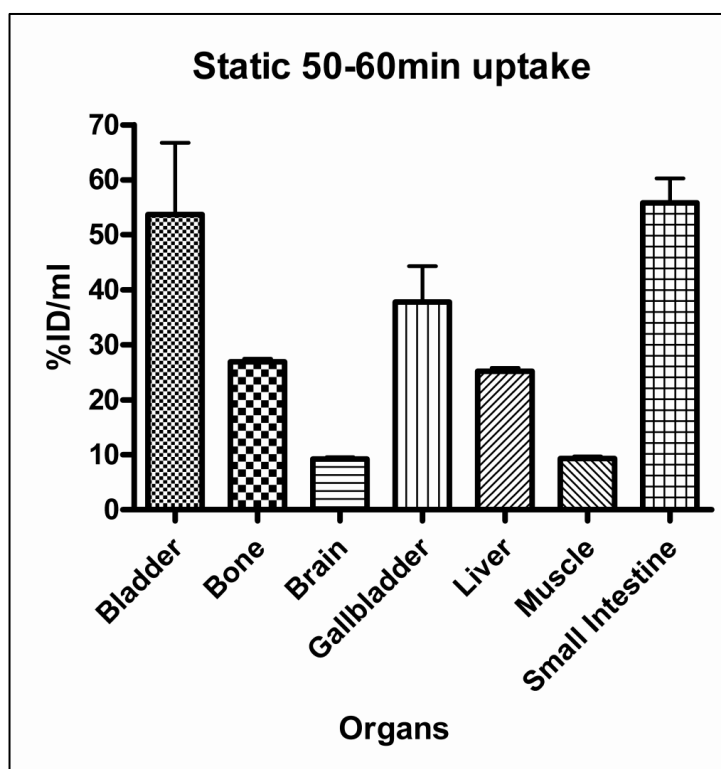


Figure 4-14: Uptake of [^{18}F]LA036 in non-tumour bearing mice organs from static (50-60min) scan.

Uptake is noted within bone (in keeping with defluorination), liver, small intestine and bladder in keeping with metabolism and excretion of tracer.

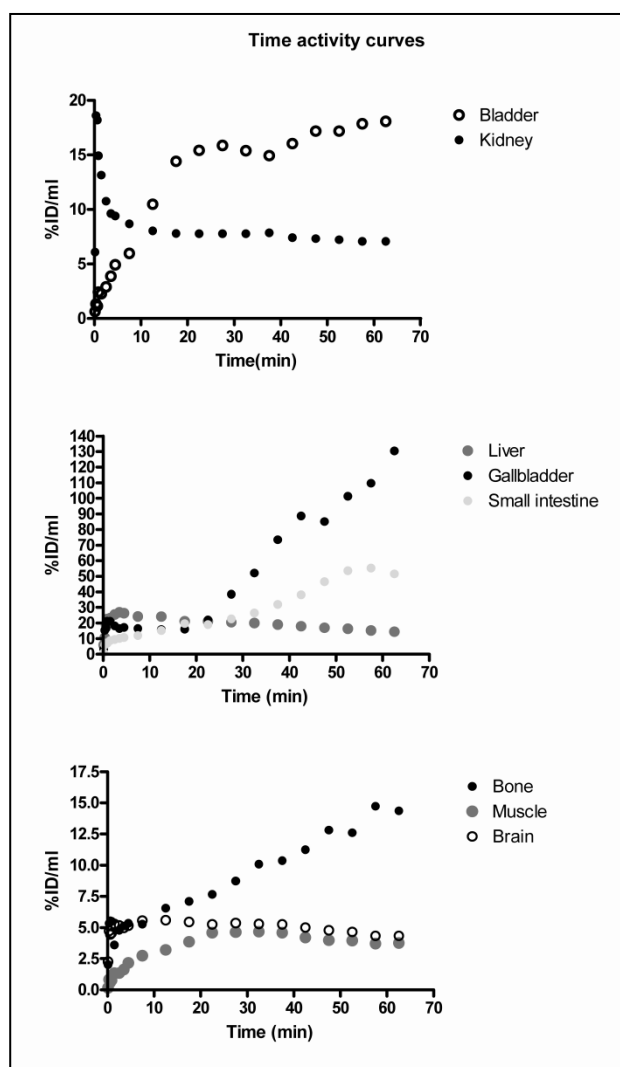


Figure 4-15: Time activity curve (TAC) of uptake of [^{18}F]LA036 in organs of non-tumour bearing mice.

The TAC of excretion (A), metabolism (B) and defluorination (C) are defined. Representative TACs of non-tumour bearing mice.

4.5.9 Biodistribution of LA036

Non-tumour bearing mouse organs were extracted following injection with LA036. In keeping with data observed in the PET image data, ROI analysis and TAC curves, highest uptake was noted in the liver, small intestine, bone and urine (Figure 4.16).

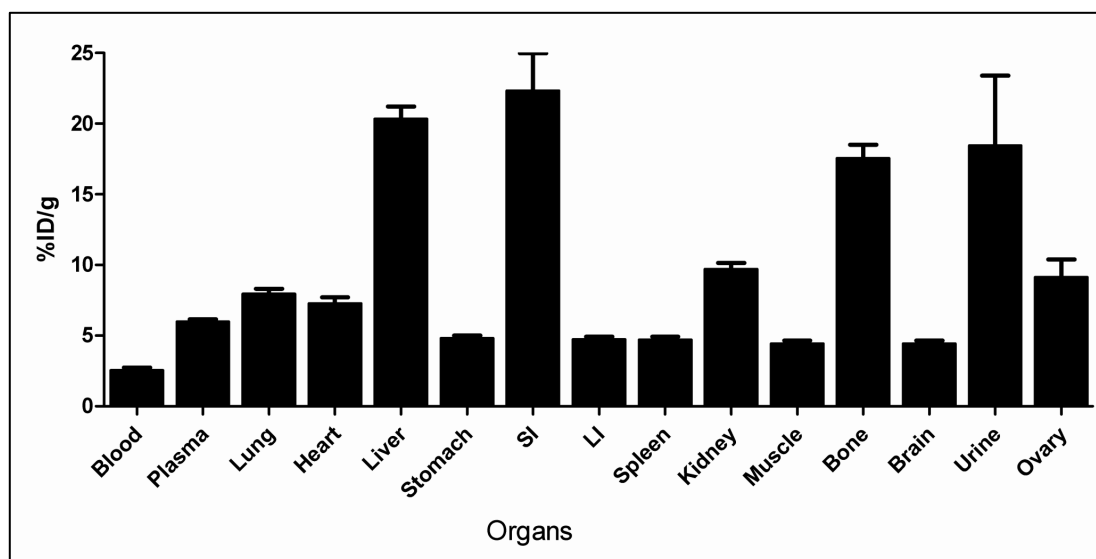


Figure 4-16: Biodistribution of [¹⁸F]LA036 in non-tumour bearing mice.

Organs were extracted after 60 min uptake of LA06 and organ radioactivity was normalized to organ weight.

4.5.10 Uptake of LA036 in T47D tumours.

T47D tumours were grown to a maximum volume of 100mm³ in SCID/beige mice. Analysis of uptake of radiotracer LA036 was assessed in PR+ve T47D tumours. PET imaging with T47D xenografts revealed uptake in the tumour with AUC₀₋₆₀ 498.79±29.7 and NUV₆₀ 7.75±0.60, relative to muscle (AUC₀₋₆₀ 423.62±26.3, NUV₆₀ 6.63±0.01) (Figure 4.17). This was further correlated to biodistribution studies in tumour-bearing xenografts.

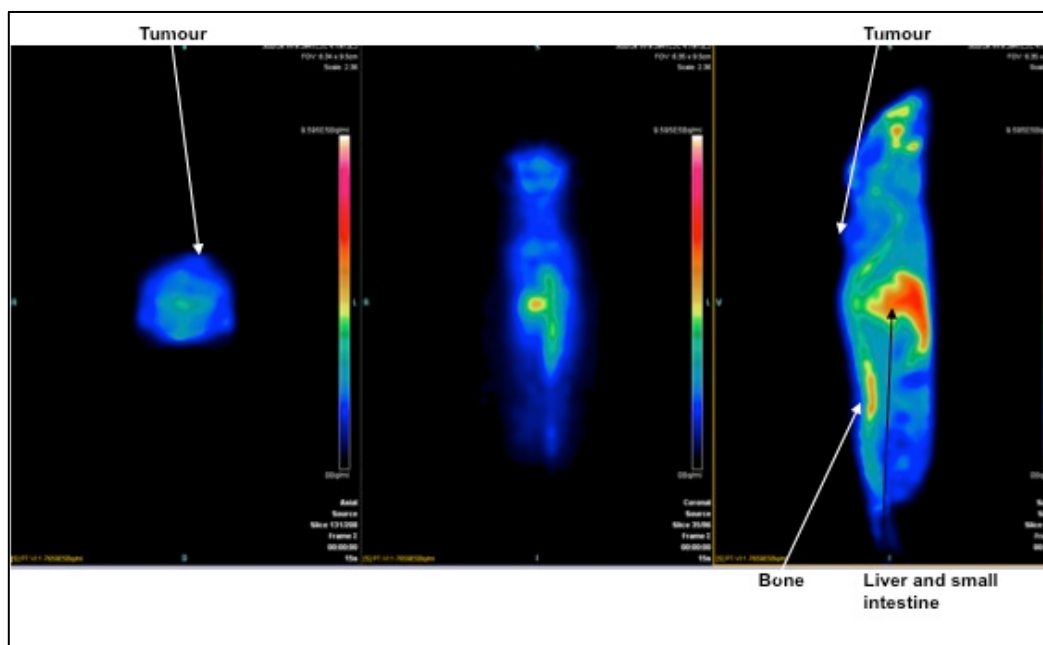


Figure 4-17: PET image of T47D xenograft with LA036.

Low uptake is noted in the tumour whilst high uptake is noted in the bone, liver and small intestine in keeping with deflourination and metabolism respectively.

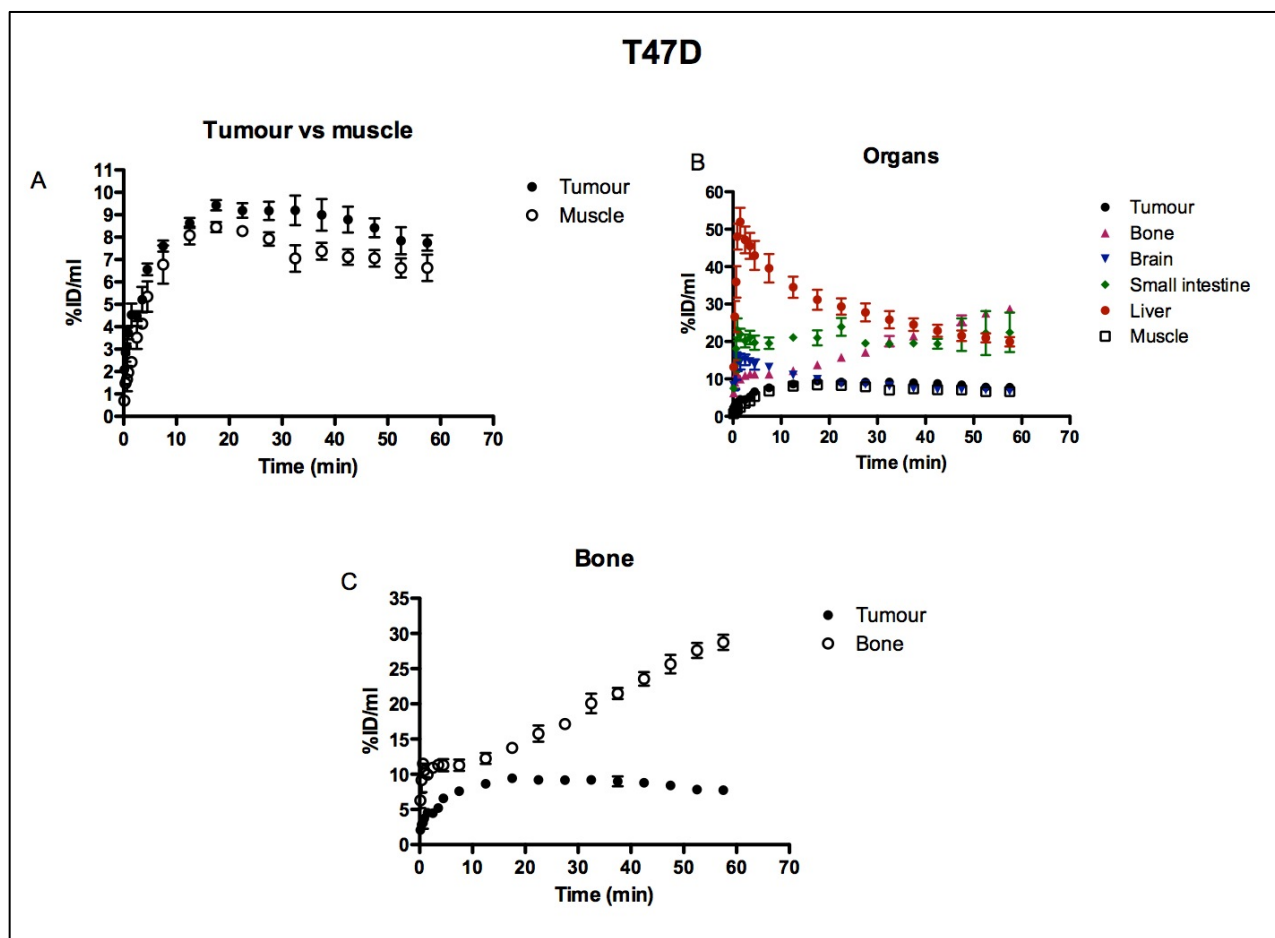


Figure 4-18: Time activity curve of LA036 in T47D tumours.

A. Uptake in tumour relative to muscle. B. Uptake in Tumour versus all organs demonstrating routes of metabolism and excretion. C. Defluorination observed in bone.

4.5.11 FDG uptake in T47D tumours

A comparison study was undertaken to compare T47D xenograft tumour uptake by [^{18}F]FDG compared to [^{18}F]LA036 (Figure 4.19). There was a 7-fold higher uptake with [^{18}F]FDG compared to [^{18}F]LA036 (Figure 4.20). However, although uptake was higher with FDG, it does not provide information on the functional nature of PR, which would be required to ascertain response to endocrine therapy.

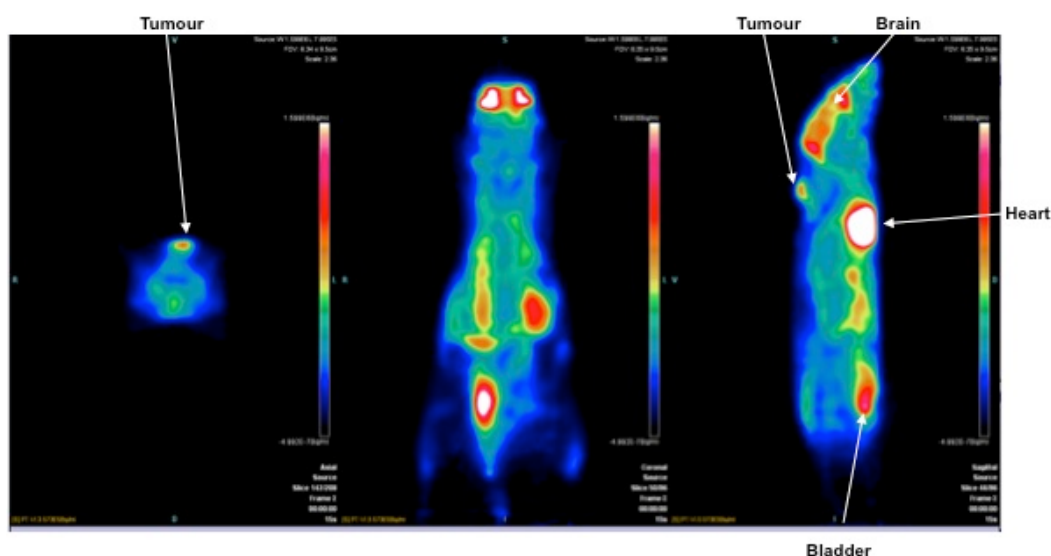


Figure 4-19: FDG uptake in T47D tumours.

T47D tumour xenografts are avid for FDG. Specific uptake is seen in the tumour, heart and brain. Renal elimination is also seen.

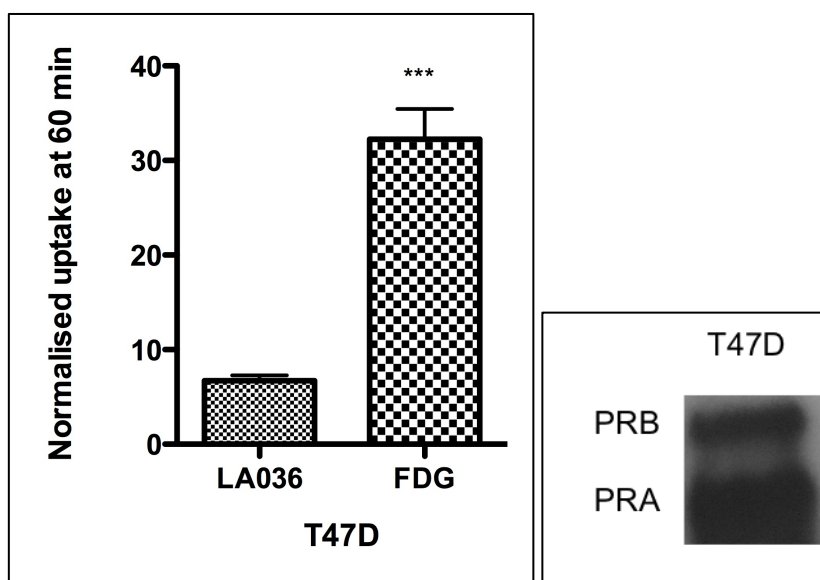


Figure 4-20: Comparison of [^{18}F]FDG uptake versus [^{18}F]LA036 and protein expression of tumour

Higher uptake with FDG is noted in T47D tumours compared to the PR specific LA036. *** $P < 0.001$. Protein analysis of T47D tumour xenograft reveals expression of PRA and PRB.

4.5.12 Metabolism of LA036 in vivo.

Unfortunately, our labelled Tanaproget-derivative [^{18}F]LA036 proved to be unstable *in vivo*, with both additional radioactive compounds present after 30 minutes post-injection in various organs, with noticeable and progressive increase in bone uptake over time. Metabolism was assessed in non-tumour bearing mice.

To confirm the hypothesis that bone uptake was a result of [^{18}F]LA036 defluorination, a metabolite assay was conducted by taking fractions at 60 min of selected organs and analysing *via* radio-HPLC (High performance liquid chromatography) (Figure 4.21). In each sample, apart from the reference compound (parent $R_t = 5.5$ minutes), [^{18}F]fluoride ($R_t = 2$ minutes, by comparison with [^{18}F]fluoride direct from the cyclotron under the same

chromatographic conditions) and a second metabolite ($R_t = 4$ minutes) were seen. In particular, circulating plasma contained more than 50% of the activity as [^{18}F]fluoride at 60 min, this supports the notion that defluorination correlates with bone uptake. A summary of the proportions of radio-analytes are shown (Table 4.3)

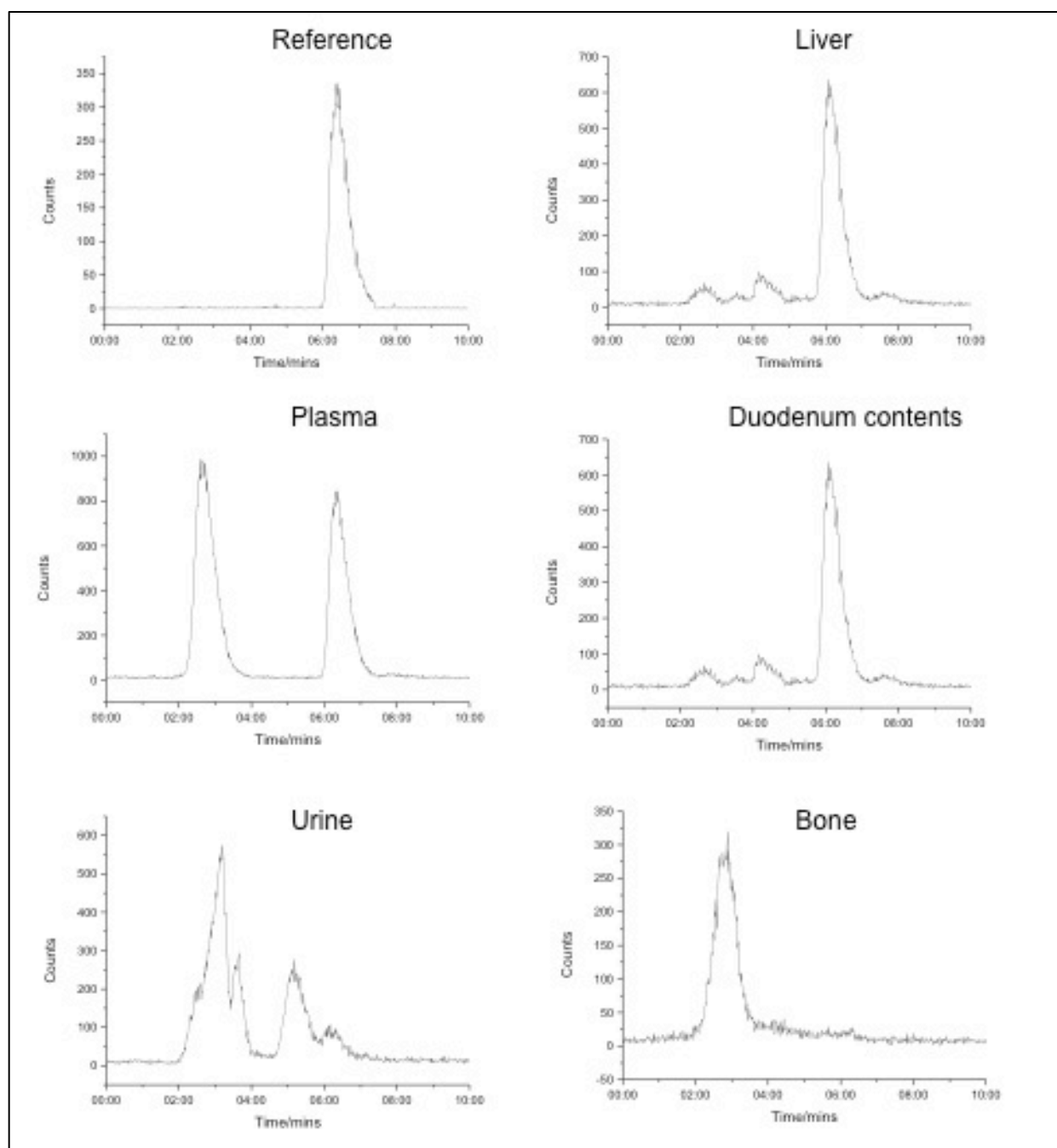


Figure 4-21: Metabolism of LA036 in vivo.

Radioactive high performance liquid chromatography (HPLC) analysis depicting metabolism of LA036 in tissues including plasma, liver, duodenum contents, urine and bone. This metabolic profile is consistent with defluorination of LA036.

Table 4-3: Fraction of [¹⁸F]LA036 remaining after 60min (n=3 for each sample)

Sample (n = 3)	[¹⁸F]Fluoride/%	Metabolite/%	[¹⁸F]LA036%
[¹⁸F]LA036 reference	-	-	99 ± 1
[¹⁸F]Fluoride reference	99 ± 1		
Urine	65 ± 3	25 ± 2	10 ± 4
Plasma	55 ± 5	-	45 ± 5
Liver	13 ± 3	5 ± 1	82 ± 2
Small intestine contents	8 ± 4	11 ± 4	81 ± 2

Although the radioligand [¹⁸F]-LA036 was noted to have high cellular uptake as well as relative binding affinity *in vitro* compared to other cell models that express high PR, *in vivo* studies have failed to show uptake of the tracer in a PR+ve model of T47D tumours. This is due to defluorination of the tracer and high metabolic instability. In addition, cell uptake studies have shown that the tracer is readily washed out within 5-10 minutes of uptake. These factors contribute to low uptake of this tracer in the tumour model.

4.6 Discussion

Radiolabelled hormones (radioligands) can be taken up selectively by tumour receptors and therefore utilised as tumour imaging agents. The radioligand readily provides information on the location of the tumour and functional status of the receptor in tumours. [108]. The Progesterone Receptor (PR) is an oestrogen-regulated gene and a PR analogue suitable for radiolabelling would be useful for imaging PR and thus functional activity of the pharmacological modulation of ER expression. Steroid-based analogues have thus far studied have shown limitations with poor receptor specificity, rapid metabolism and unfavourable biodistribution. The core structure of a putative PR imaging agent to be studied was based on tanaproget, a non-steroidal progestin that has been derivatised for radiolabelling by the Katzellenbogen group [107, 109]. Further the tanaproget compound [^{18}F]FPTP has been tested *in vivo* with successful imaging of PR in uterus and ovaries of oestrogen-primed rats [106], with good receptor specificity and biodistribution characteristics similar to [^{18}F]FENP and [^{18}F]FFNP.

Based on the tanaproget core, the compounds developed for PR imaging were small molecule non-steroidal PR binding agents and have been synthesised to probe structure-activity relationships (SAR) relative to PR. On this basis, they should have high affinity for PR and low cross-reactivity with other receptors including the androgen receptor (AR) and glucocorticoid receptor (GR).

The carbonyl compound LA036 was shown to bind PR with a low nanomolar affinity and the relative binding affinity was 140% to that of progesterone binding to PR. Due to constraints in our laboratory of radiolabelling thiocarbonyl compounds, LA036 compound was utilised for further development of a PR based radioligand. Further, as fluorine-18 has the most favourable physical and nuclear properties, LA036 was eventually radiolabelled with ^{18}F for further *in vitro* and *in vivo* testing.

In vitro analysis confirmed increased uptake of LA036 by PR+ve cell lines over PR-ve cell lines. Further the specificity of LA036 for PR was confirmed by blocking studies which showed that uptake in PR could be blocked by substrates selective for the PR LBD. In addition, cross-reactivity assays were in keeping with the tanaproget profile, showing negligible interaction of LA036 with other steroid hormone receptors ER, AR and GR. These favourable attributes of LA036 led to *in vivo* testing. Retention of the tracer by the cells was explored in cell-based assays with washout of tracer noted between 5-60 minutes following change of media to radioactivity-free media. The lack of retention of the radiotracer was noted even in the presence of ABC transporter inhibitors. It is possible that in the *in vitro* setting, the binding of small molecule ligands is transitory and competitive inhibition occurs by progesterone being produced in oestrogen-rich conditions.

In vivo studies of [^{18}F]LA036 was investigated in normal female mice and in T47D xenograft bearing mice. Tumour uptake was poor which was likely due to poor metabolic stability over the imaged-time frame. In addition, the RBA of LA036 relative to progesterone was lower than other compounds such as R5020 (775%>Progesterone) and ORG2058 (200%>R5020) [100]. The low RBA can account for low target to normal tissue visualisation. Further it may be possible that low nanomolar affinity for PR was sufficient *in vitro* but insufficient for investigation of PR expression *in vivo*. The presence of circulating endogenous steroid hormones could also lead to competitive inhibition of the radioligand *in vivo*. Extensive defluorination was observed at 60min. The biodistribution profile of the radioligand revealed similar characteristics to the other PR radiotracers with metabolism noted in the liver, gall bladder and small intestines. Of note, there was no uptake of radioligand noted in adipose(fat) tissue as has been the case with other steroid based analogues, therefore, a non-steroidal analogue is preferable for further development.

In comparison, the T47D tumour xenograft model displayed high uptake in tumour with [^{18}F]FDG and provided high contrast between tumour and normal tissue with non-specific uptake noted in the heart and bladder. Therefore, for future studies of other PR tracers, the T47D xenograft can be adequately utilised as a tumour model.

In summary, we have synthesised analogues of the non-steroidal progesterone, tanaproget and subsequently labelled with ^{18}F after selection of

the most appropriate compound for imaging of the progesterone receptor. *In vitro* the compound LA036 showed affinity for PR with a high RBA, high specificity for PR and little cross-reactivity to other steroidal receptors such as AR, GR and ER. Despite these favourable characteristics, *in vivo*, low tumour uptake was noted with high uptake in the metabolising and excreting organs. The compound was also unstable *in vivo* with additional metabolites and defluorination in bone. Further work is needed to produce a more suitable radioligand, which could eventually be utilised in clinical studies.

4.7 Future work

Certainly a non-steroidal based PR imaging agent would be ideal due to low cross-reactivity with other steroid hormones. We need to identify other similar progestins that demonstrate high affinity for PR and investigate some of the problems encountered with ligand retention, metabolic instability and defluorination.

Chapter 5 Apoptosis imaging in pre-clinical breast cancer with the radiotracer ^{18}F [ICMT-11]

5.1 Introduction

5.1.1 Apoptosis

Apoptosis is the organised, energy-dependent form of cell death and is a process essential for maintenance of tissue homeostasis for eliminating unwanted cells during growth and differentiation. Evasion of apoptosis is classed as a hallmark of cancer [110] and cancer treatments including chemotherapy, radiotherapy as well as molecular targeted treatments induce tumour death through apoptosis [111]. The morphological changes of apoptosis are characterised by cell shrinkage, membrane blebbing, and compartmentalisation of intracellular contents into small membrane bound apoptotic bodies. At the molecular level, the apoptosis pathway is activated by the death receptor extrinsic pathway or the mitochondria-directed intrinsic pathway which converge on the activation of effector caspases (caspases-3,-6 and -7 that execute apoptosis [112]. Activation of caspase 3, termed executioner caspase, leads to degradation of DNA to fragments of multiples of 200 base pairs along with cleavage of key cellular structures and their subsequent disposal [113]. Another biochemical change of apoptosis is the rapid externalisation of phosphatidylserine residues from the inner to the outer surface of the plasma membrane. These biochemical occurrences can be utilised to image apoptosis non-invasively following cancer treatments. Moreover, apoptosis imaging would enable detection of tumour biochemical changes evolving within hours of therapy, in contrast to changes in tumour size which can take many months to detect on RECIST.

5.1.2 [¹⁸F] ICMT-11

The imaging of the activated executioner caspase-3 is an attractive strategy for apoptosis imaging. Small molecule PET radioligands based on the isatin-5-sulfonamides class of caspase-3 inhibitors have been developed to image the committal stage of programmed cell death. A library of compounds with the isatin-5-sulfonamide scaffold screened for activated caspase-3 inhibitory affinity led to the development of ¹⁸F-(S)-1-((1-(2-fluoroethyl)-1H-[1,2,3]triazol-4-yl)methyl)-5-(2(2,4-difluorophenoxy)methyl)-pyrrolidine-1-sulfonyl)isatin ([¹⁸F]ICMT-11), with subnanomolar affinity for activated caspase-3, reduced lipophilicity, favourable biodistribution and clearance profiles as well as facile radiolabelling [114], hence providing a novel reagent to non-invasively image apoptosis.

5.1.2.1 Proof of concept

In preliminary studies [114] of assessment of the radiotracer [¹⁸F]ICMT-11, *in vitro* binding assays of murine fibrosarcoma RIF-1 and human pulmonary carcinoma cells LNM35 were conducted in response to topoisomerase inhibitor etoposide and alkylating agent cisplatin. Cellular uptake of [¹⁸F]ICMT-11 was higher in cells treated with chemotherapy and this was correlated to high cellular caspase-3/7 activity. Further, *in vivo* studies showed 2-fold activity of [¹⁸F]ICMT-11 uptake on PET 24h following chemotherapy, compared to controls. This was confirmed by tumour time versus radioactivity curves and other PET imaging variables (NUV60, AUC, FRT) as well as voxel-wise analysis of the PET data and immunohistochemistry. High background uptake was noted in the liver, small intestine, kidney and urine

demonstrating rapid distribution to tissues and routes of elimination in addition rendering it difficult to interpret this tracer in intra-abdominal malignancies due high uptake in this region. In summary, initial studies for the novel apoptosis tracer confirmed its sensitivity and specificity for caspase activation and therefore apoptosis.

5.1.2.2 Response to cytotoxic and mechanism based treatments

In a study to define the optimal time point to detect apoptosis following cytotoxic therapy, murine B cell lymphoma 38C13 cells were treated with cyclophosphamide (CPA) [115]. Peak caspase-3 activation occurred 24 hours after treatment, increased 2-fold and decreased at 48hours. This reflects the early and transient nature of the apoptotic process. The observation of heterogeneous spatial distribution of [^{18}F]ICMT-11 and correlation to patches of activated caspase-3 and TUNEL staining, led to voxel-wise analysis. This entailed the sorting of all voxels according to their intensities in the VOI and therefore the development of PVIS (PET-based voxel-intensity sorting) histograms. Rather than quantify radiotracer uptake of the whole tumour, PVIS histogram analysis allowed for tumour heterogeneity by evaluation of the highest intensity voxels within the VOI, and therefore provide a more representative analysis of the biological process of apoptosis. A right shift of PVIS histogram 24h and return to baseline at 48h after treatment was in keeping with the transient nature of this apoptotic process.

[^{18}F]ICMT-11 specificity was further investigated with a small molecule second mitochondria-derived activator of caspases (SMAC) mimetic, birinapant, that

activates caspase and therefore apoptosis [115]. A longitudinal study of treatment of colon cancer HCT116 tumour and breast cancer MDA-MB-231 tumours were treated with birinapant. [^{18}F]ICMT-11 uptake associated apoptosis was noted to be 1.5 fold increased as early as 6h following treatment, compared to baseline. However, at 24h and 48h post-treatment, levels of [^{18}F]ICMT-11 uptake decreased to baseline levels, reflecting the rapid and dynamic activation of caspase-3 with birinapant. Histological analysis confirmed apoptosis with birinapant treatment with increase cleaved caspase-3 and TUNEL staining. This model therefore suggests that apoptosis detection depends on the cell type, mechanism of apoptosis-inducing agent (cytotoxic versus mechanism-based) as well as determination of optimum drug delivery time, dose and schedule to determine the true pharmacodynamic assessment of non-invasive apoptosis imaging. In addition, voxel intensity sorting analysis of [^{18}F]ICMT-11 PET data informs on the heterogeneity of detection of caspase-3 foci.

5.1.2.3 Apoptosis versus necrosis

[^{18}F]ICMT-11 uptake was evaluated in two lung cancer models both *in vitro* and *in vivo* and compared to molecular biochemical measures of apoptosis[116]. The non-small cell lung cancer cell lines PC9 and A549 are characterized by low DNA-damage repair protein ERCC1 expression and mutation in EGFR in PC9 cells while A549 cells exhibit high ERCC1 and wild-type EGFR. Flow cytometry confirmed apoptosis as the mechanism of cell death in PC9 cells and necrosis in A549 cells. At 24h and 48h following chemotherapy, PET imaging of both tumour lines did not reveal any significant

difference in uptake between control and treatment defined by tumour activity curves and normalised uptake values at 60min. In voxel-wise analysis of the PET data, there was a clear shift in the histograms of PC9 cells over the 48h time course. There was a 1.5 fold increase in the number of voxels that depicted high intensity. Therefore, PET-based [^{18}F]ICMT-11 uptake, required voxel-wise analysis to determine apoptosis in a pre-existing necrosis model of PC9 cells (confirmed by histology). In contrast, A549 tumours, although responsive to carboplatin by inhibition of tumour growth, did not exhibit apoptosis in biochemical or histological analysis and thus not detectable by PET with [^{18}F]ICMT-11.

In summary, the use of [^{18}F]ICMT-11 has been investigated in several tumour models including lymphoma, breast and lung cancer with reference to single agent chemotherapeutic drugs and targeted molecular therapy. Apoptosis is an early event in the response to cancer therapy and early time points of apoptosis have been identified prior to changes in tumour volumes. Histogram analysis has contributed to identifying areas of heterogeneity within areas of apoptosis.

Clinical breast cancer treated with neoadjuvant chemotherapy is utilised to reduce tumour size prior to surgery to improve surgical cosmesis as well as reduce risk of recurrence in patients who also have nodal disease. FEC (5FU, Epirubicin and cyclophosphamide) chemotherapy is used in the neoadjuvant and adjuvant setting in breast cancer treatment and is associated with better clinical outcomes in those achieving pathological complete remission [117]. In node positive disease, docetaxel chemotherapy is further added to this

combination to improve clinical outcomes [118]. In the neoadjuvant setting, FEC chemotherapy is given for a total of 3-6 cycles and response assessed clinically by palpable tumour measurement or by radiological ultrasound. However, early determination of response by apoptosis imaging would enable early response assessment in determining responsive tumours and more importantly unresponsive tumours for which treatment could be modified and further correlate this to clinical outcomes.

The aim of the study was to determine the temporal nature of apoptosis in breast cancer models *in vivo*. The use of combination chemotherapy to assess apoptosis in breast cancer *in vivo* models has not been studied. Thus utilising orthoptic xenograft models of breast cancer, the triple negative MDA-MB-231 cell line xenografts were treated with FEC chemotherapy and a longitudinal time point study (24-96h) was performed in an attempt to determine the optimum time point of apoptosis for biomarker evaluation using the apoptosis PET tracer [¹⁸F]ICMT-11. In addition, correlation of apoptosis by tissue analysis of TUNEL and cleaved caspase-3 as well as determination of proliferation by Ki67 immunohistochemistry was performed.

5.1.3 Study methodology

Triple negative breast cancer is most sensitive to chemotherapy and therefore the MDA-MB-231 (ER-, PR-, HER2-) cell line was the most suitable model for the purpose of this study. Balb/c nude mice were implanted with subcutaneous injection of 5×10^6 MDA-MB-231 and treated with intraperitoneal FEC chemotherapy once xenograft tumour size reached 80-100mm³. PBS was used as vehicle. MDA-MB-231 xenografts were treated with combination chemotherapy with FEC as per the clinical treatment. The usual dose for clinical breast cancer is 500mg/m² of 5FU and Cyclophosphamide and 100mg/m² of Epirubicin given intravenously. However, we optimised our treatment utilizing previously published data on combination chemotherapy given in vivo. FEC chemotherapy included 5FU 65mg/kg, Cyclophosphamide 130mg/kg and Epirubicin 10mg/kg and animals were treated with 0.01ml/g of combination treatment given as a single intra-peritoneal injection. PBS was used as vehicle. Following chemotherapy, the animals were imaged with [¹⁸F]ICMT-11 PET tracer at the following time points 24h, 48h, 72h, 96h and tissues were further excised for bio-distribution analysis and sectioned for immunohistochemistry staining.

5.2 Results

5.2.1 PET imaging

Tumour uptake by [^{18}F]ICMT-11 was not noted in the vehicle treated tumours but was noted at all other time points suggesting that apoptosis occurs within 24h of chemotherapy and can continue for up to 96h post-treatment (Figure 5.1). The NUV_{60} (%ID/ml) of the tracer at baseline was 0.203 ± 0.15 , while the NUV_{60} of the tracer at 24h, 48h, 72h and 96h time points was 0.789 ± 0.07 , 0.831 ± 0.15 , 0.903 ± 0.25 and 0.757 ± 0.1 , respectively, suggesting that as early as 24h post chemotherapy, there was an increase in apoptosis which peaked at 72h and started to decline by 96h (Figure 5.2).

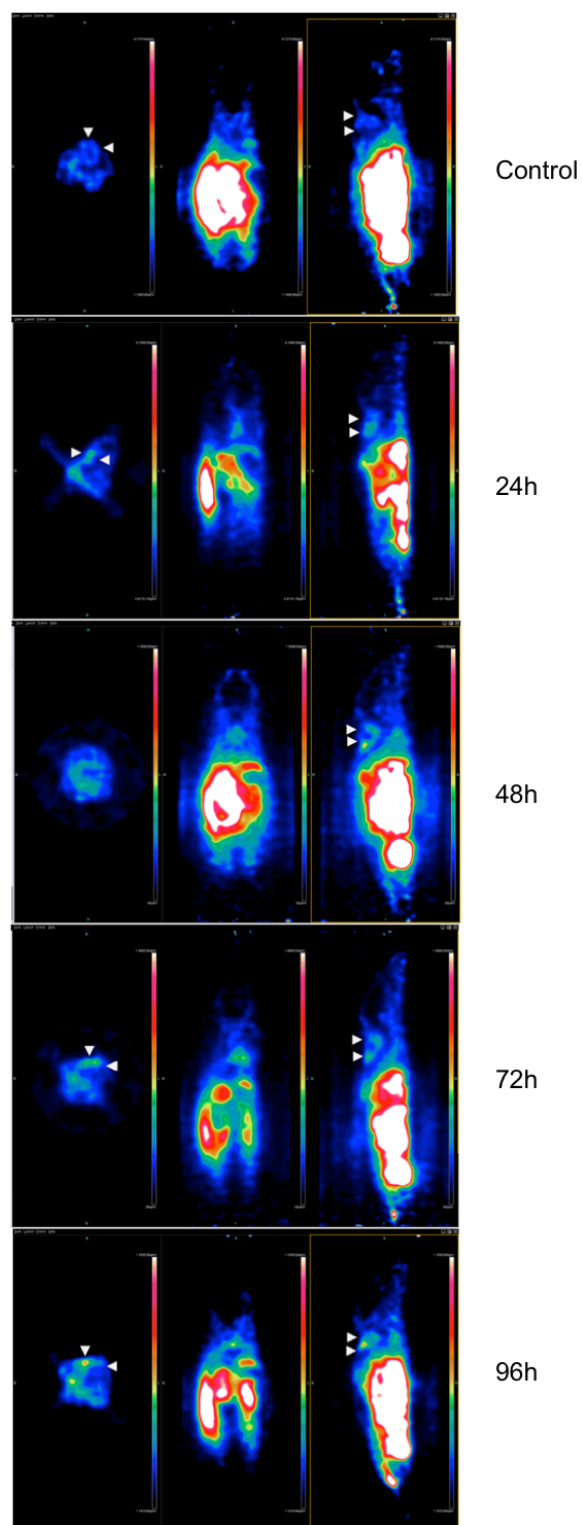


Figure 5-1: Apoptosis imaging with [^{18}F]ICMT-11.

MDA-MB-231 xenografts in Balb/c nude mice were treated with vehicle (PBS) i.p. or combination chemotherapy with FEC i.p. and imaged at time points of 24, 48, 72 and 96h post treatment. Apoptosis signal was not noted at baseline but increase in apoptosis was noted at all other time points. Tumour heterogeneity is also noted with areas of necrosis demonstrating reduced uptake. Arrows represent tumour in coronal (left image) and sagittal sections (right image)

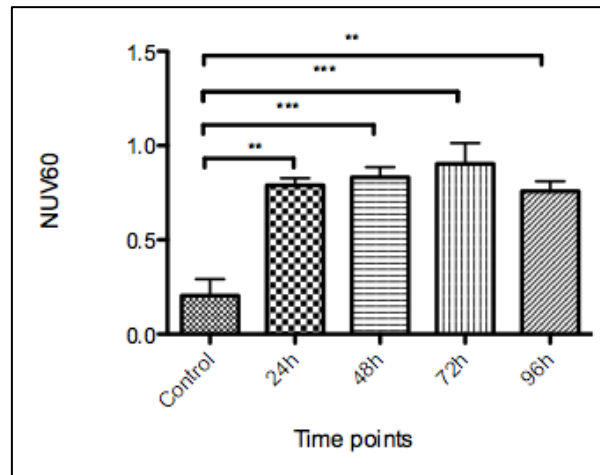


Figure 5-2: NUV₆₀ of treatment time-points.

Tumour uptake from static PET images were normalised to whole body uptake (Bq/ml) to provide normalized uptake values at each time point. NUV₆₀ increased as early as 24h post chemotherapy and peaked at 72h followed by a decrease at 96h. Data are mean±SEM (n=5) for each time point. 24 and 96h ** P<0.01. 48 and 72h treatment ***, P=0.0001.

5.2.2 PET Voxel Intensity Sorting

Radiotracer uptake is assessed on the whole tumour. However, intrinsic heterogeneity of uptake occurs within tumours that exhibit both apoptosis and necrosis. The PET based voxel intensity sorting (PVIS) allows for characterisation of VOI by sorting through voxel intensities. In this way, the PVIS histograms generated enable a right shift (higher intensity voxels) to be visualised in keeping with apoptosis tracer uptake over the time periods assessed in this study. Further, the highest voxel intensities (95th percentile) are more indicative of high radiotracer retention than the voxel mean and this has been quantified to be more representative of the biological process of apoptosis.

In this tumour model, the PVIS histograms demonstrate a right shift in keeping with apoptosis occurring 24h after treatment and sustained up to 96h following treatment with a peak of apoptosis visualised at 72h post chemotherapy treatment (Figure 5.3).

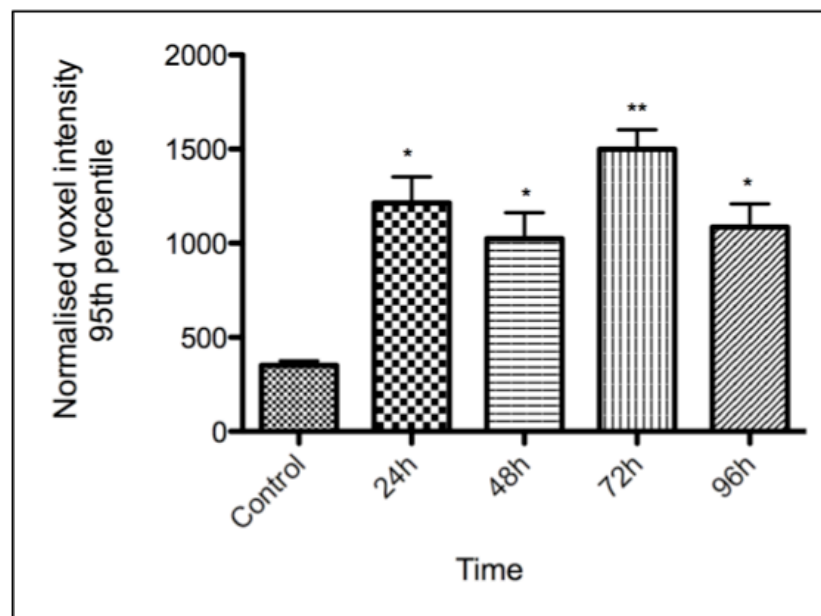
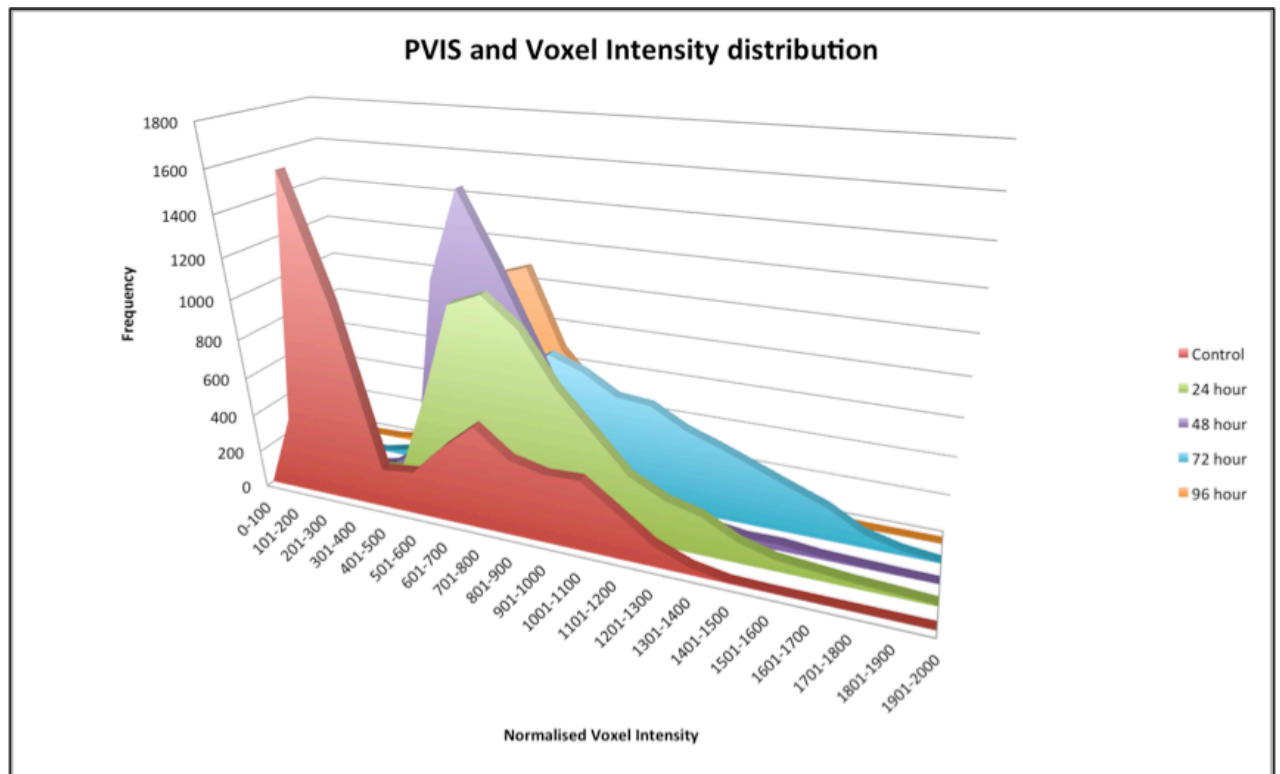


Figure 5-3: PVIS and voxel intensity distribution analysis.

The voxel intensities within the volumes of interest (VOI) were computed and expressed as histogram plots of normalised voxel intensity versus frequency of number of voxels. These are further represented statistically by expressing the 95th percentile utilizing Prism v5.0 software (Graphpad) over the imaging time points of between 24h-96h post chemotherapy. Mean ± SD (n=4-6 animals per time point). *P=0.05, **P=0.005

5.2.3 Biodistribution

Tumours were excised following injection of radioactivity to assess uptake in tumour tissue (biodistribution) and correlate this to the PET imaging data. Apart from uptake at 24h post chemotherapy, uptake in the tumour was not noted at the other time points (Figure 5.4). The radioactivity was normalised to injected dose and weight of tumour. It is possible that as the tumours were also sectioned for immunohistochemistry staining, the full extent of biodistribution was not fully analysed and therefore differs from the PET data.

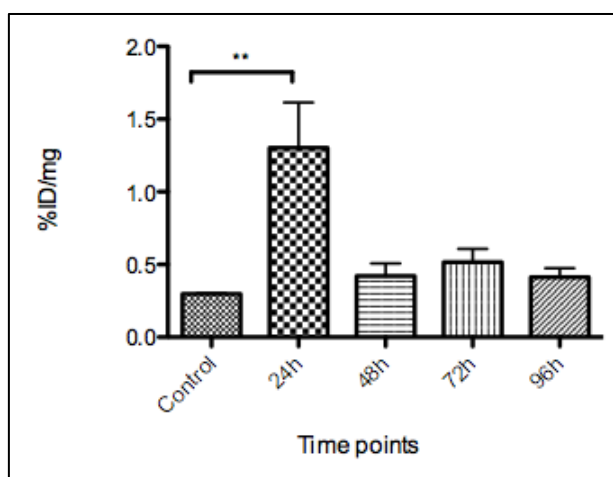


Figure 5-4: Biodistribution of tumour tissue.

Tumours excised after each imaging time point were assessed for radioactive uptake using a gamma counter and activity was normalised to injected dose and weight of tumour (%ID/mg). Data shown are mean $\pm$ SEM (n=3) for each time point. Control vs 24h **P=0.0014.

The effect of combination chemotherapy was also assessed on tumour volumes and body weight of the animals to assess toxicity of the regimen on the animals. The tumours did not reduce in size following FEC chemotherapy at the time points of 24-96h but there was a reduction in weight (3-8% change) after treatment of less than 10% indicating that the regimen was reasonably tolerated [36]. This was notable at all time points accounting for treatment effect within 24 hours of treatment (Figure 5.5).

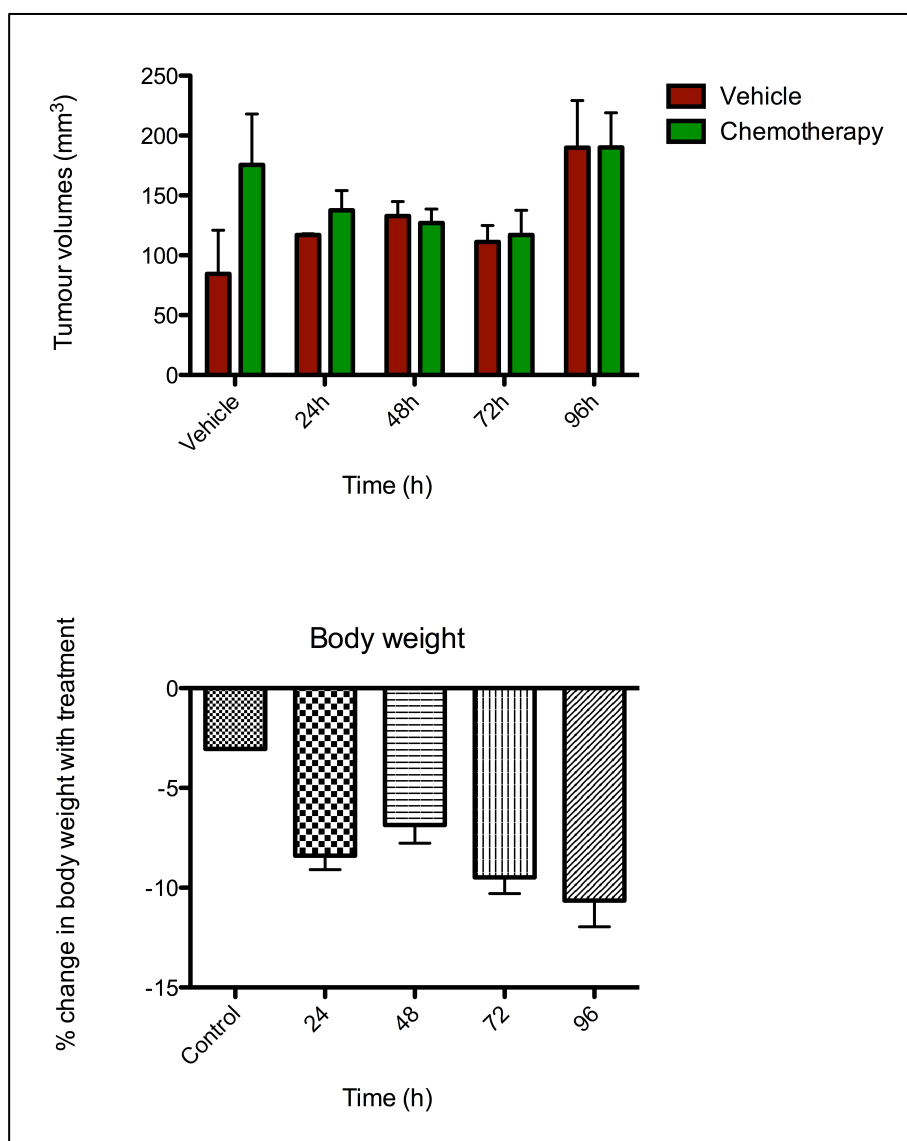


Figure 5-5: Tumour volumes and body weight in response to FEC chemotherapy.

Tumour volumes did not change in response to chemotherapy. Tumour volumes were assessed prior to treatment and at imaging time points of 24-96h suggesting a cystostatic response after examining the proposed timelines. Although body weights did not reduce by more than 10% there was some toxicity associated with the treatment with changes in body weight noted of between 3-8%.

5.2.4 Immunohistochemistry

Following imaging studies with [^{18}F]ICMT-11, tumours were excised and analysed by immunohistochemistry for proliferation with the Ki67 antibody. After treatment of MDA-MB-231 xenografts with chemotherapy, there was up to a 3-fold decrease in Ki67 score from 24-96h suggesting response to chemotherapy (Figure 5.6) with the most significant decrease at 96h post chemotherapy treatment.

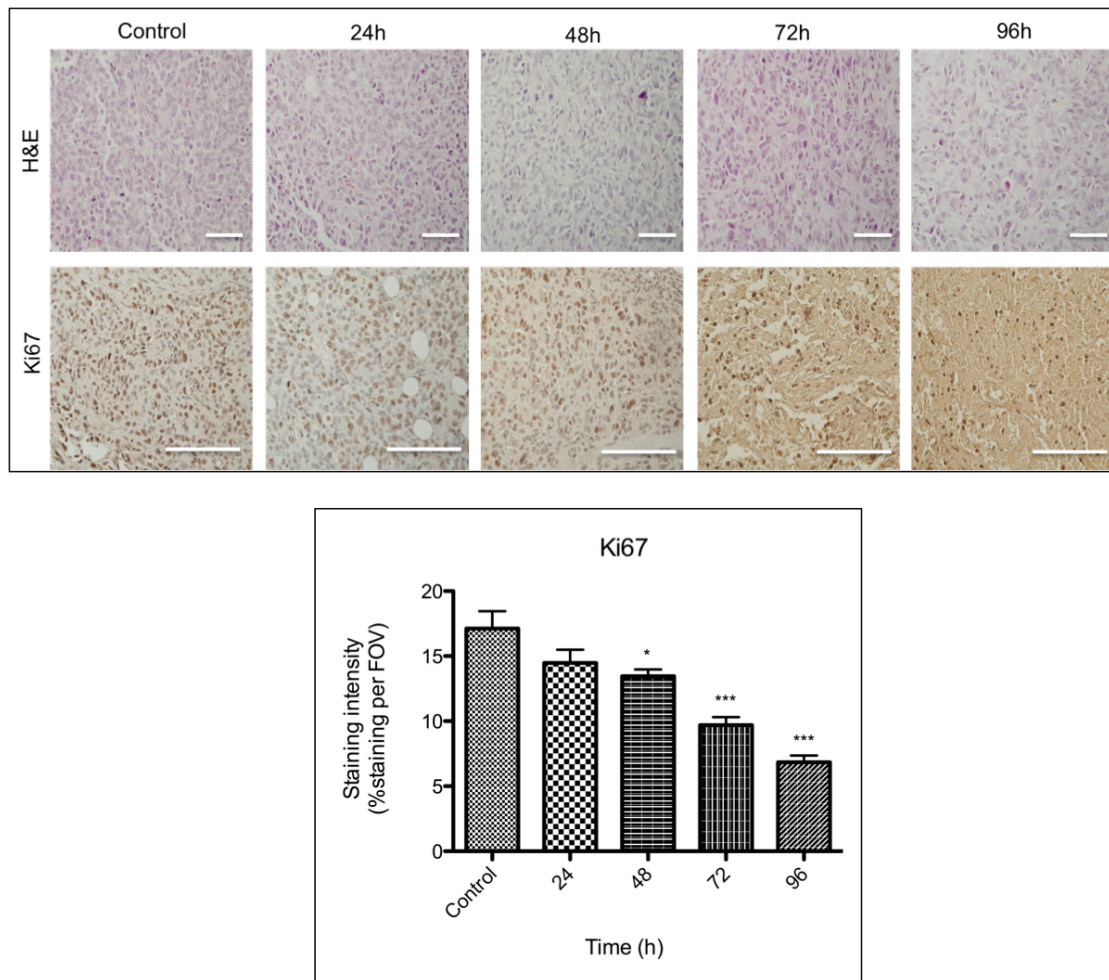


Figure 5-6: Ki67 and H&E staining on xenografts.

Representative images of MDA-MB-231 tumour tissue excised following FEC chemotherapy and stained for H&E as well as the proliferation marker Ki67. Graphical representation of Ki67 score of $n=4$ tumours (Mean $\pm$ SD). *, $P<0.05$, *** $P<0.005$. $n=3$ tumour sections with 5-10 random field of view (FOV) per section. Images of H&E-stained sections were acquired at 100 $\times$ magnification, Ki67 images acquired at 400 $\times$ magnification. Scale bar=100 μ m

5.2.5 Immunofluorescence

Xenograft tumours excised following PET imaging studies were preserved and sectioned. Following this, tumours were analysed for apoptosis immunofluorescence markers of TUNEL and caspase to correlate to the apoptosis radiotracer [¹⁸F]ICMT-11. Immunofluorescence was then quantified using Image J[®] software to obtain percentage staining per field of view (Fig 5.7). TUNEL and caspase staining of the xenografts revealed significant apoptosis at 48 hours following chemotherapy with both methods displaying a 3- fold ($P<0.001$) increase in apoptosis over the control arm.

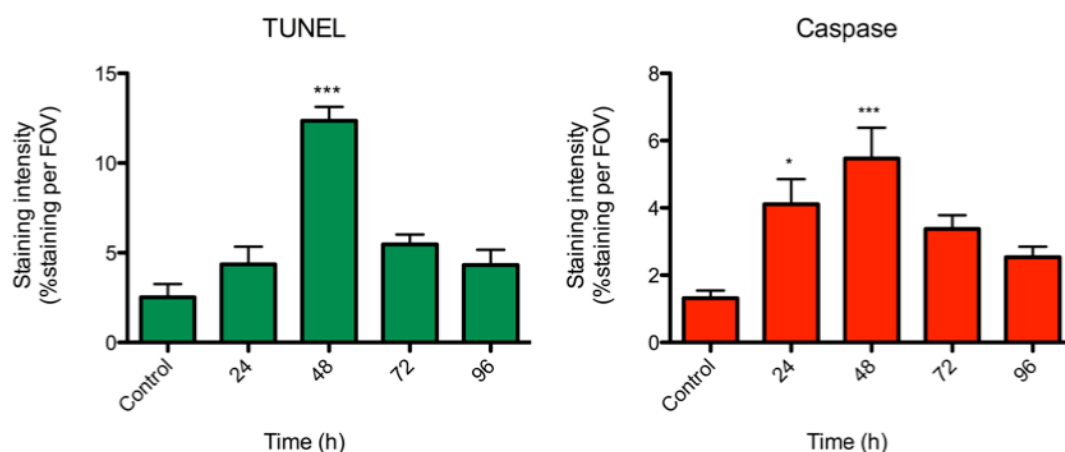
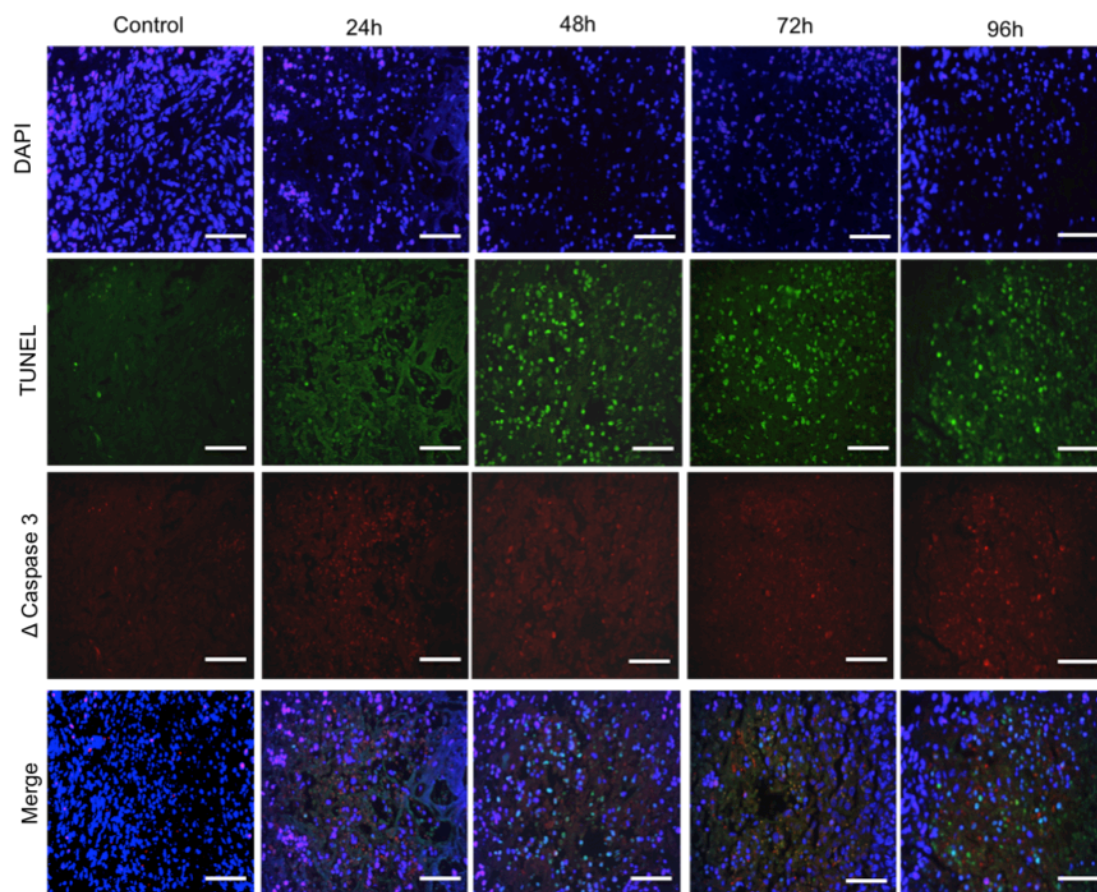


Figure 5-7: Tumour caspase and TUNEL immunochemistry analysis.

Tumour samples were removed after PET imaging and processed for immunohistochemical analysis including staining for TUNEL (DNA fragmentation) and cleaved caspase 3. Representative sections of tumour are shown stained for DAPI, TUNEL and cleaved caspase 3. Staining intensities for TUNEL and cleaved caspase 3 were obtained using Image J software and expressed as percent staining per field. Data are mean±SD. *P<0.05; ***P<0.001. . n=3 tumour sections with 5-10 random field of view (FOV) per section. 400x magnification. Slide bar=100 μm

5.3 Discussion.

Apoptosis imaging provides a surrogate and early marker of treatment response. Histological assessments of treatment response can be invasive and problems of tumour sampling, intratumoural heterogeneity and non-uniform drug delivery can be negated with non-invasive imaging. We have developed a novel apoptosis tracer [^{18}F]ICMT-11 that binds to capsase-3 with subnanomolar affinity and has been utilised in pre-clinical and clinical models to determine its efficacy as a biomarker. In initial studies of this tracer, lymphoma models were utilised [114], subsequently it has been assessed in breast, colorectal [115] and lung cancer models[116]. Responses to treatment with a mechanistic inducer of apoptosis were noted as early as 6 hours following treatment and these effects were transient [115]. However, in cytotoxic treated models of lung cancer, apoptosis was noted at 48 hours post chemotherapy [116]. The aim of this study was to assess the temporal effect of apoptosis in a breast cancer model treated with combination chemotherapy to reflect the treatment of clinical breast cancer. In clinical breast cancer, neoadjuvant and adjuvant chemotherapy is provided as combination chemotherapy. In this study, triple negative breast cancer model MDA-MB-231, xenografts were selected to be treated with combination chemotherapy (FEC) to assess response by apoptosis imaging and correlate this to biological and immunohistochemical data.

Apoptosis in relation to cytotoxic chemotherapy is known to occur at early time points after initiation of chemotherapy and is noted to be a transient effect. In this study, the xenografts were therefore treated with chemotherapy and

imaged between 24-96 hours later to determine the temporal nature of this effect. On imaging of the tumours post chemotherapy, there was an increase in uptake of the tracer noted 24 hours after chemotherapy and sustained up to 96 hours following chemotherapy. This effect is in contrast to studies published previously where uptake of the radiotracer is noted up to 48 hours post treatment [115, 116]. These studies however, utilised single agent treatments rather than combination chemotherapy. To account for tumour heterogeneity and to determine the intratumoural regions of high [^{18}F]ICMT-11 uptake, the PET-based voxel intensity sorting histograms also confirmed apoptosis occurring up to 96 hours following treatment with a peak of apoptosis occurring at 48-72 hours following treatment.

Further, the imaging data were correlated to immunohistochemistry. Ki67 the proliferation marker decreased following chemotherapy and was sustained at all time points. The apoptosis stains with caspase 3 and TUNEL showed significant 3-fold increase at 48 hours post chemotherapy but were also increased at all other time points compared to baseline. In relation to changes in tumour volume and animal weight following chemotherapy, the chemotherapy effect was cytostatic. This is contrast to the previous studies [114, 115] where decreases in tumour volumes were noted within 24h of chemotherapy, however, the tumour models utilised were lymphoma models and are more sensitive to chemotherapy than breast cancer models. Toxicity was limited with the combination chemotherapy.

Biodistribution data from the tumour samples did not show correlation to the imaging data with change in tracer uptake noted only at 24 hours post chemotherapy. Certainly, this does need further validation as the other parameters in this study favour the imaging data with sustained levels of apoptosis noted from between 24-96hours post treatment with chemotherapy. This could also be due to biodistribution samples also being sectioned for immunohistochemistry stains and this appears to have introduced sampling errors, which will need to be examined further.

In summary, the determination of the ideal time point for determining apoptosis in cancer treatments relies on a number of factors including tumour type and heterogeneity, treatment type, whether cytotoxic or targeted, length of treatment and host factors. Certainly, in this study, the effect of combination chemotherapy in a breast cancer model, is noted with increased apoptosis seen as 24h and maintained up to 96h post treatment. This has not been reflected in the previous studies using single agent chemotherapy. Toxicity was limited and correlation to imaging data was provided with Ki67, TUNEL and caspase-3 staining. The imaging data was further validated by the PVIS histograms.

5.4 Future Work

This study provided an insight into the timeline of apoptosis in a breast cancer model treated with combination chemotherapy. Apoptosis occurs within 24h of chemotherapy and seems to be sustained up to 96 hours following chemotherapy. It would be necessary to pinpoint a more precise time point for clinical trials so as to avoid repeated exposure of patients to radioactive isotopes.

Further work would require the investigation of other tumour models such as ER, PR+ve and HER2+ve models. In addition, a longitudinal study of the same xenograft model over the timeline would eliminate xenograft bias. However, the toxicity of the treatment should be considered. In clinical breast cancer, treatment is provided intravenously and a further similar study would require intravenous treatment rather than intraperitoneal treatment.

Chapter 6 Apoptosis imaging in Clinical Breast cancer following treatment with neoadjuvant chemotherapy

6.1 Introduction

The use of neoadjuvant, primary or pre-operative chemotherapy in locally advanced breast cancers is currently used to downsize large, operable tumours and therefore enable breast-conserving surgery as well as treating occult systemic disease [119, 120]. In addition, neoadjuvant chemotherapy does not alter local recurrence rates [120] and survival rates are not compromised [121]. Certainly clinical response to neoadjuvant chemotherapy is associated with improved survival [121]. It also provides a model to study the effects of chemotherapy on cellular and molecular changes within the tumour. In order to assess response to therapy, clinical, radiological and pathological endpoints are utilised. However, patients who either have stable disease or progressive disease during neoadjuvant chemotherapy need to be identified early to modify treatment regimes. Biological or molecular imaging markers can therefore be used as predictive markers of response and provide surrogate assessments of long-term outcome. Biological markers such as Ki67 (proliferation) and apoptosis have been used to predict clinical response and survival in neoadjuvant breast cancer patients.

6.2 Apoptosis radiotracers used in clinical studies.

6.2.1 Annexin V

The first imaging biomarker that was used to evaluate apoptosis clinically was ^{99m}Tc -annexin-V – detected by scintigraphy. This tracer binds with high affinity (sub nM) to phosphatidylserine groups that are exposed on the cell surface during the process of apoptosis. In an initial study of 15 patients with lung cancer, breast cancer and lymphoma treated with just one cycle of chemotherapy, increased radiolabelled-annexin-V uptake was observed in 7 patients and was associated with a favourable response on conventional imaging [122]. ^{99m}Tc -Annexin-V scintigraphy was also used to determine response to radiotherapy in 11 patients with follicular lymphoma. A baseline scan pre-radiotherapy and a post treatment scan 24 hours after radiotherapy were conducted. In 10 patients, increase in ^{99m}Tc -Annexin was noted following treatment, which correlated to cytologic assessment of apoptosis and clinical outcome [123]. In another study of 33 patients, ^{99m}Tc -Hynic-rh-Annexin V scintigraphy (TAVS), was used to predict response to therapy. Patients with lymphoma, leukaemia, head and neck cancers were scheduled for chemotherapy, radiotherapy or combined chemoradiotherapy depending on tumour type. Baseline and early post-treatment scans were conducted. Increase in TAVS post-treatment was associated with complete or partial tumour response while no significant increase was noted in uptake of radiotracer in tumours that exhibited stable or progressive disease. [124]. These studies reflect the effect of chemotherapy and radiotherapy on apoptosis and the utilisation of annexin-V apoptosis imaging as a surrogate

biomarker of therapy and response. The limitations of radiolabelled annexin-V imaging have been poor specificity for apoptosis over necrosis, slow-clearance from non-targeted tissues and problems with radiolabelling [125].

6.2.2 ^{18}F -ML-10

The *Aposense* family of compounds is a novel class of small non-peptide molecules which have been developed to discriminate viable from apoptotic cells. In the early stages of the apoptotic process, these compounds selectively bind to membranes and accumulate in the cytoplasm of apoptotic cells [126]. The *Aposense* radiotracer ^{18}F -ML-10 has successfully completed a healthy volunteer study showing favourable dosimetry, biodistribution, stability and safety profile, with binding to apoptotic sites in the testes of men observed [127]. Furthermore, in a trial to assess response of brain metastasis to whole-brain radiotherapy, patients underwent ^{18}F -ML10-PET scans at baseline and after 10 fractions of radiotherapy. Conventional MRI was performed 6-8 weeks after radiotherapy. A positive correlation was noted between early changes in ^{18}F -ML-10 and late changes in tumour anatomical dimensions [128].

6.3 Assessment of apoptosis parameters in clinical studies and relevance to clinical response.

In a preliminary study [129] to assess apoptosis in early breast cancer by preoperative chemotherapy, 27 patients were treated with epirubicin, cisplatin and fluorouracil as well as doxorubicin and cyclophosphamide. Tru-cut biopsies were done 7 days before chemotherapy and 24 hours after treatment. Apoptosis index was assessed with the in-situ end-labelling assay. Overall, there was a significant increase in apoptosis noted in the 24h biopsy specimens. 9 of 13 clinical responders showed a greater than 50% increase in apoptosis index, whereas the 3 of 4 non-responders showed no significant change in the apoptosis index.

A further study in neoadjuvant chemotherapy in breast cancer evaluated apoptosis changes in 11 patients treated with paclitaxel every 2 weeks [130]. Apoptosis was examined by fine needle aspiration (FNA) at 24, 48, 72 and 96h after first paclitaxel dose. For the majority of the 11 women, there was a 3-6 fold increase in apoptotic activity compared to baseline at some point during the first 4 days after initial chemotherapy treatment. Interestingly, the apoptotic curves were different for each patient, but all demonstrated apoptotic response over 4 days. In addition, the cumulative apoptotic response i.e. sum of relative changes in apoptotic index for 1-4 days after chemotherapy showed an inverse correlation to proportion of cancer volume, despite variable time points for apoptosis detection in individual patients.

Further studies have examined the timing of apoptosis and its clinical relevance. In a study by Archer et al [131], 66 breast cancer patients had core biopsies prior to and 24 hours post chemotherapy. These were analysed for Ki67 (proliferation) and apoptosis utilising TUNEL technique. A significant increase in apoptosis and a decrease in Ki67 was noted on tumour tissues 24 hours post-chemotherapy but this did not correlate to clinical response or survival. Buchholz et al [132] analysed biopsies of 25 breast cancer patients pre-chemotherapy, 24h and 48h after chemotherapy. Apoptosis was quantified using TUNEL staining. Apoptosis levels at 48 hours were significantly higher in tumour with a complete pathological response or minimal residual disease.

Tiezzi et al [133] similarly addressed the relationship between apoptosis and clinical response. 44 patients underwent chemotherapy for breast cancer in the neoadjuvant setting. Apoptosis was determined using morphological criteria. Patients completed a median of 3 cycles prior to surgery, but those that had no clinical response after 2 cycles of chemotherapy were subjected to an incisional biopsy. Median apoptotic index was 0.82 (0.12-2.49) before chemotherapy and 1.61 (0.32-4.31) post chemotherapy. A significant increase in apoptotic rate was observed in patients who had a clinical response. However, in patients who did not show a clinical response, there was no change observed in the apoptotic rate suggesting that chemo-resistance is associated with failure of apoptosis.

Burcombe et al [134] evaluated apoptosis and Ki67 proliferation before, during and after neoadjuvant chemotherapy for primary breast cancer. 27 patients underwent FEC (5-fluorouracil, epirubicin and cyclophosphamide). Biopsies were obtained pre-chemotherapy, at day 21 (before 2nd cycle of chemotherapy) and at surgery. Apoptosis was visualized by TUNEL staining. Median apoptotic indices at baseline were 1.92% (0.23-5.4%), at day 21 1.69 (0.33-11.2%) and 2.19% (0.9-4.9%) at surgery. The percentage change in apoptotic index at day 21 was compared to pre-treatment levels. Apoptotic index decreased in 50%(10/20 patients), increased in 45% (9/20) and was unchanged in one patient. This was similarly seen comparing baseline biopsy with surgical specimens, with a reduction in apoptotic index in 8 of 17 patients analysed. Interestingly, there was an increase in apoptotic index between day 21 and surgery in 10 of 18 specimens analysed. Median apoptotic index as well as changes in apoptosis index from baseline did not correlate with clinical and pathological responders and non-responders. However, logistic regression analyses identified increased apoptotic index at day 21 as a significant predictor of pathological response.

Li et al [135] have described molecular imaging techniques with [¹⁸F]-FDG PET in breast cancer patients undergoing neoadjuvant chemotherapy. 45 patients underwent 3 cycles of neoadjuvant chemotherapy. PET/CT was performed prior to neoadjuvant chemotherapy and before surgery. Breast tissue samples were obtained during initial diagnosis and at surgical resection. PET variables assessed were tumour to non-tumour radioactivity ratio (T/N) and apoptosis determined by TUNEL staining. The mean T/N pre-chemotherapy was 3.32 and post chemotherapy was 2.31 with a mean

reduction from baseline of 31%. The mean apoptotic index pre-chemotherapy was 2.81 and post chemotherapy was 17.31 with a mean apoptotic index change of 14.34%. A statistically significant correlation was noted between T/N reduction rate and the mean apoptotic index suggesting that PET/CT, through reduced glucose uptake post-chemotherapy, can be correlated to apoptosis and predicts response to chemotherapy.

Kanjer et al [136] also analysed archival material from 106 patients. Diagnostic and surgical specimens were examined for Ki67 (immunohistochemistry) and apoptosis (TUNEL assay). A significant decrease in Ki67, apoptotic index and growth index (Ki67/Apoptotic index ratio) were noted in surgical specimens post chemotherapy. Patients had been treated with 3-4 cycles of anthracycline-based chemotherapy. Only high baseline Ki67 was associated with pathological response.

This suggests that variables such as type of chemotherapy, patient factors, molecular pathways and timing of apoptosis evaluation can all contribute to biomarker assessments. [¹⁸F]ICMT-11, which is a molecular marker for apoptosis imaging may provide a non-invasive and early imaging biomarker of correlation to caspase 3/7 activity in patients with breast cancer undergoing neoadjuvant chemotherapy.

The safety and biodistribution of [¹⁸F]ICMT-11 has been validated in healthy volunteer trials [137]. [¹⁸F]ICMT-11 was evaluated in 8 healthy volunteers. No serious tracer-related adverse events were reported. Organs receiving the highest absorbed dose included liver, gallbladder wall, small intestine, large

intestine wall and bladder wall. Elimination was both via the renal and hepatobiliary systems.

6.4 [¹⁸F]ICMT-11 uptake in breast cancer patients following neoadjuvant chemotherapy: Trial design

[¹⁸F]ICMT-11 is an isatin analogue, developed as a novel PET radiotracer for studies of apoptosis. Preclinical studies have demonstrated subnanomolar affinity to caspase 3, and validated the potential for imaging apoptosis in xenograft models. A first-in-man study showed that the agent was well tolerated with acceptable dosimetry. This is the first study of this agent to measure the effect of chemotherapy on radiotracer uptake in patients. The main objectives of the study were to determine the optimal time-point for imaging apoptosis post-chemotherapy and compare the results with immunohistochemistry assessments at the same time-points.

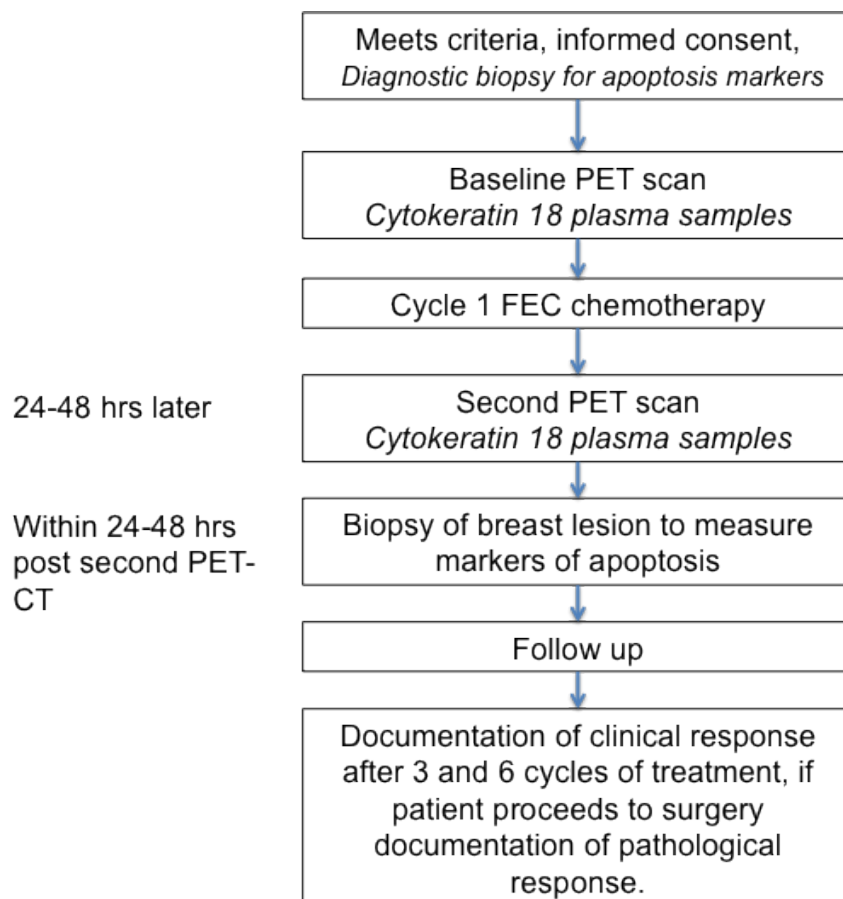
Patients with primary breast tumour lesions of $\geq 15\text{mm}$ and who were suitable for treatment with neoadjuvant chemotherapy with FEC (5FU, Epirubicin, Cyclophosphamide) underwent dynamic PET scans for 66mins 30seconds following intravenous injection of [¹⁸F]ICMT-11 with a mean activity of $340.82 \text{ Mbq} \pm 20.76$ and Specific Activity range of $447.014\text{--}5128.34 \text{ GBq}/\mu\text{mol}$. PET scans were conducted prior to chemotherapy and 24h-2 weeks post-chemotherapy. A breast biopsy was also obtained within a few hours of the 2nd PET scan to correlate apoptosis in the breast tissue utilising TUNEL and Caspase 3 staining by immunohistochemistry as well as to determine the proliferation index Ki67. For analysis of the dynamic PET imaging, volumes of

interest were drawn manually and analysed using Analyze software® and parameters such as Standardised Uptake Volume (SUV) mean and maximum were obtained as were tumour to breast and tumour to muscle ratios (TBR and TMR respectively).

Subjects who had a diagnosis of breast cancer and due to have neoadjuvant chemotherapy, were selected by oncologists according to the study entry criteria. For patients who consented to the study, diagnostic biopsy was obtained from Imperial College NHS Trust pathology department.

Patients underwent 2 visits for the PET study: at baseline, prior to start of neoadjuvant chemotherapy and between 24hours and 2 weeks (specifically 24-48h and 1-2 weeks) post chemotherapy. At the PET imaging visit, each subject received a single dose of [^{18}F]ICMT-11 injection with a maximum administered ^{18}F activity of 370MBq. Subjects had a single dynamic emission acquisition centred on the area of interest for approximately 1 hour. Arterial blood samples were acquired for metabolite analysis to allow optimisation of kinetic modelling and improve our understanding of [^{18}F]ICMT-11 pharmacokinetics. A core biopsy of the tumour was performed under ultrasound guidance within 24h of the second PET scan to examine immunohistochemical and molecular markers of apoptosis (TUNEL and cleaved caspase-3). Plasma samples were taken at baseline and at the time of the second scan for measurement of markers of apoptosis including M30 and M65 fragments of cytokeratin 18. Cytokeratin 18 (CK18) is an important component of the epithelial cytoskeleton and is released into blood either in the intact form (M65) or the cleaved forms (M30) during apoptotic or necrotic

cell death. The M65 and M30 assays have been shown to be important predictive and prognostic biomarkers in carcinomas and have been described in response to chemotherapy in breast cancer [138]. However, it has also been reported in other clinical situations such as sepsis, hepatitis and ischaemic heart disease [139], and therefore careful evaluation in relation to carcinomas is required.



6.5 PET imaging

Images were acquired on a Siemens Biograph 6 TruePoint PET/CT scanner (with TrueV; extended field of view) with 21.6-cm axial and 60.5-cm transaxial fields of view. On the day of the PET scan, a transmission scan was obtained using a low dose attenuation CT prior to the administration of [^{18}F]ICMT-11 for each subject. CT settings: 130 kV; 30 effective mAs; pitch, 1.5; slice thickness, 5 mm; rotation time, 0.6 s; and effective dose [ED], 2.5 mSv). Patients underwent a PET image acquisition of the area of interest beginning at the time of [^{18}F]ICMT-11 administration. This dynamic image acquisition lasted approximately 66 minutes in 1 bed position.

6.6 Chemotherapy

Patients due neoadjuvant chemotherapy were treated with combination fluorouracil, epirubicin, and cyclophosphamide (FEC) chemotherapy as per local hospital guidelines and treated in their local oncology unit.

6.7 Intervals between scans and treatment

Following the baseline scan, the second [^{18}F]ICMT-11 PET scan was acquired between 24h-2 weeks of the first administration of chemotherapy

6.8 Dose administered

In order to obtain diagnostic-quality images, subjects received target administered activity of 300MBq of [^{18}F]ICMT-11 injection, with a maximum administered activity of 370 MBq and a minimum of 140MBq.

Subjects were administered a single i.v. dose of 1-20 mL of [^{18}F]ICMT-11 injection over a period of 30 seconds. [^{18}F] ICMT-11 was injected through a cannula (or indwelling venous catheter) with the subject lying in a supine position.

6.9 Blood sampling

Blood samples obtained from subjects with breast cancer for counting of ^{18}F in blood/plasma and for measuring ^{18}F metabolites in plasma, were drawn from arterial blood. The use of arterial blood is necessary to obtain measurements suitable for kinetic modelling. Continuous arterial blood sampling at 5 mL/min was performed for the first 10 min. Discrete blood samples were taken from baseline to 1 hour post administration for ^{18}F counting. Arterial blood samples counted for tracer activity and metabolites of [^{18}F]ICMT-11 were used to generate the blood input function used to derive the uptake tracer parameters.

Total blood radioactivity was monitored by gamma counting and plasma [^{18}F]ICMT-11 parent fraction (and metabolite, [^{18}F]ICMT-11) was determined by reverse-phase high-performance liquid chromatography with radiochemical detection (as described previously)

Plasma cytokeratin 18 samples were taken from the venous line at baseline and at the time of the second [^{18}F]ICMT-11 scan. The second core biopsy of the breast lesion was scheduled within 24h of the second PET scan.

6.10 Clinical response

Clinical response data were collected after 3 (by standard ultrasound) and 6 cycles of treatment. For patients proceeding to surgery, pathological response data was recorded in the Case reporting form (CRF)

6.11 Translational Research

6.11.1 Cytokeratins

Venous plasma was assayed for cytokeratin 18 by ELISA for M30 and/or M65 fragments, at baseline (2 samples to check for inherent variability), and at the time of the second [^{18}F]ICMT-11 scan.

6.11.2 Tissue response

A core biopsy was performed by a consultant radiologist within 24h of the second [^{18}F]ICMT-11 scan. Tissues were analysed for immunohistochemical markers of apoptosis of cleaved caspase 3, PARP and TUNEL assays.

6.12 Reconstruction of PET data

Emission data were reconstructed using the ordered subsets expectation maximisation algorithm (3 iterations and 21 subsets). The data were binned into time frames and decay corrected images were viewed using ANALYZE analysis software (version 11; Biomedical Imaging Resource, Mayo Clinic).

Volumes of interest of primary tumours and lymph nodes were manually defined to quantify the average and maximal standardised uptake value

(SUV_{av} and SUV_{max}) which is the uptake of activity in tumour normalised to injected [¹⁸F]CMT-11 dose and body surface area. Tumour to breast (TBR) and tumour to muscle (TMR) ratios were also defined.

6.13 Results

6.13.1 Patient characteristics

The scans were well tolerated in all patients with uptake of [^{18}F]ICMT-11 demonstrated in all tumour lesions. The tumours studied included ER positive and PR positive, HER2 positive and triple negative.

Table 6-1: Patient characteristics.

Patients with invasive ductal carcinoma treated with neoadjuvant chemotherapy and radiological response measured at 9 weeks post chemotherapy by ultrasound. Abbreviations: IDC, invasive ductal carcinoma; PR, partial response; SD stable disease; FEC, 5FU, Epirubicin, Cyclophosphamide.

Patient No	Age	Type of Breast cancer	LN	Size	Grade	ER/PR/HER2	Treatment	Clinical response
1	60	IDC	+	48mm	3	+/+/-	FEC	PR
2	41	IDC	-	70mm	2	-/-/+	FEC	SD
3	64	IDC	+	50mm	3	-/-/-	FEC	PR
4	54	IDC	+	70mm	2	+/+/-	FEC	PR
5	56	IDC	+	30mm	3	+/+/-	FEC	SD
6	43	IDC	+	25mm	2	+/+/-	FEC	PR
7	67	IDC	+	32mm	3	+/+/-	FEC	PR
8	45	IDC	+	18mm	3	-/-/+	FEC	PR

6.13.2 Patients studied at 24-48h after initiating chemotherapy

3 patients were in this initial cohort to determine if apoptosis could be observed at 24-48h after chemotherapy. Three different histological subtypes were analysed including ER/PR +ve patient, HER2+ve patient and patient with a triple negative breast cancer.

6.13.2.1 Patient 1

This patient is a 60 year old female with a 48mm left-sided Grade 3 ER+ve, PR+ve invasive ductal carcinoma with involvement of ipsilateral axillary lymph nodes. The patient was imaged prior to chemotherapy and within 24 hours of commencing chemotherapy (Fig 6.1). Although [^{18}F]ICMT-11 uptake was not noted in the tumour following chemotherapy, increased uptake was noted in the smaller lymph node, SUV_{av} increased from 0.37 to 0.42 and SUV_{max} from 0.89 to 1.13 (Table 6.2). Histogram analysis is in keeping with decrease in apoptosis following chemotherapy (Fig 6.2). The patient was treated with FEC chemotherapy and following 3 cycles of chemotherapy, was noted to have a partial response on ultrasound.

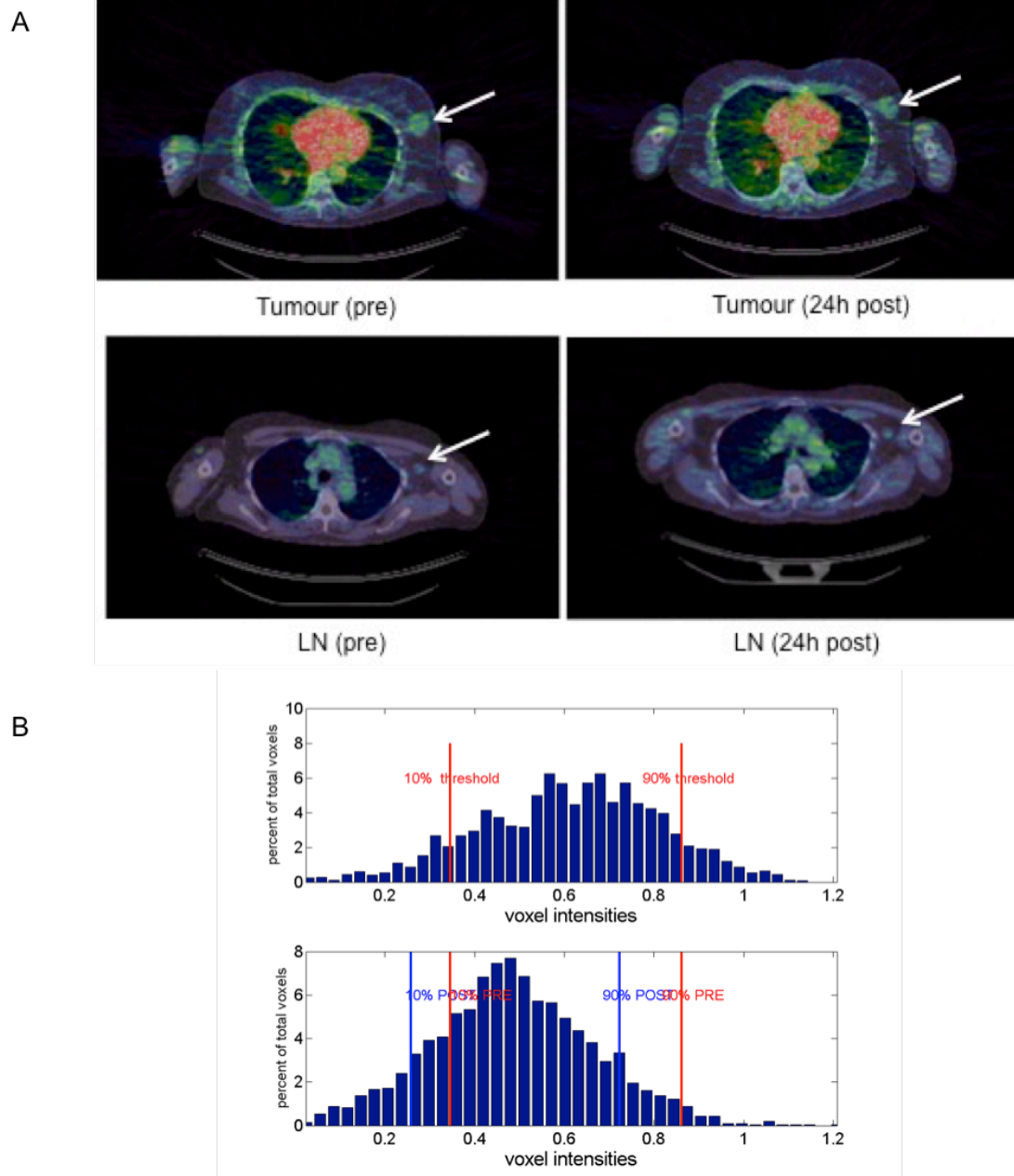


Figure 6-1: Patient 1.

Patient with ER+/PR+/HER2- 48mm Grade 3 tumour. Uptake of [^{18}F]ICMT-11 is noted on PETCT pre and 24h post chemotherapy within the tumour and axillary lymph nodes (A). Voxel analysis on histogram shows a decrease in intensity in the tumour following chemotherapy (B)

Table 6-2: Patient 1 parameters.

Tumour and lymph node (LN) SUV (standardized uptake value) have been assessed at 60min. Abbreviations TBR, Tumour to breast ratio; TMR, tumour to muscle ratio; av, average; max, maximum. *denotes increase in parameter consistent with change in apoptosis.

	Pre-chemotherapy	Post-chemotherapy
Tumour SUV_{av}	0.45	0.32
Tumour SUV_{max}	1.41	1.09
LN SUV_{av}	0.37*	0.42*
LN SUV_{max}	0.89*	1.13*
TBR_{max}	1.33	1.04
TMR_{max}	1.18	1.12

Immunohistochemistry

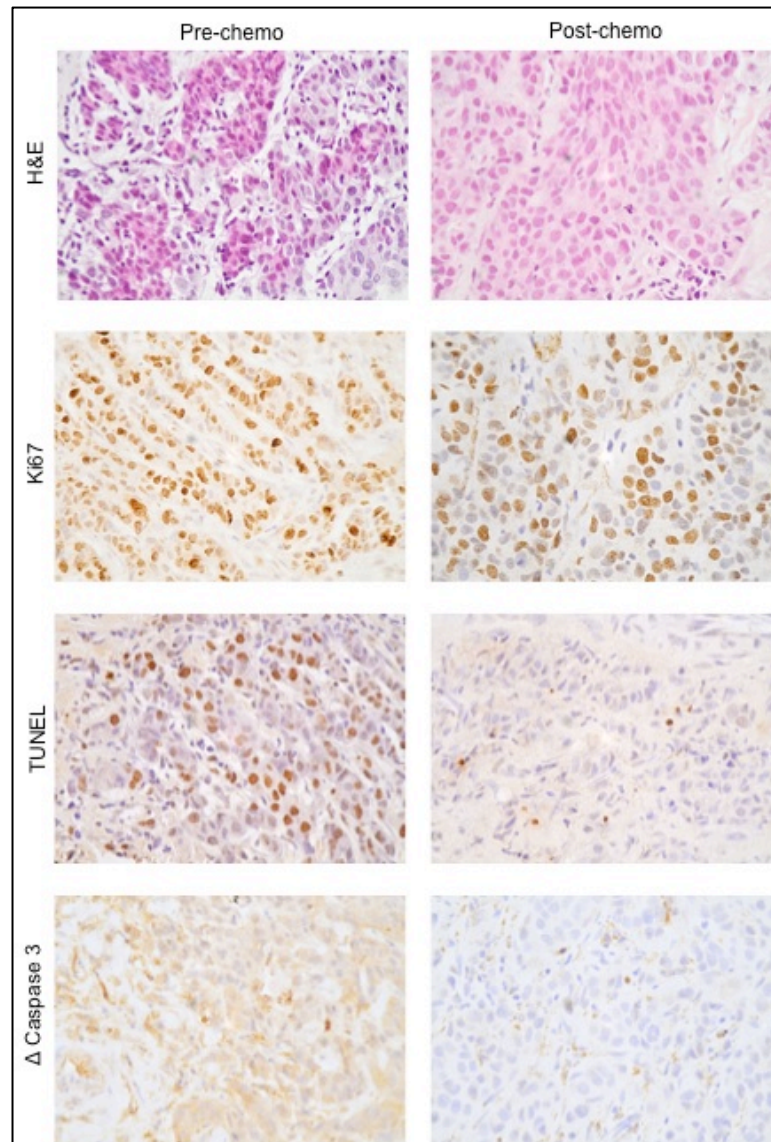


Figure 6-2: Patient 1 immunohistochemistry.

Immunohistochemistry with Ki67, TUNEL and cleaved caspase 3. Ki67 was reduced from 100 to 53% following chemotherapy, while TUNEL was increased from 21 to 36 cells. Caspase analysis was difficult due to high background staining. $n=3$ tumour sections with 5-10 random field of view (FOV) per section. 400x magnification

On immunohistochemistry analysis, the proliferation index Ki67 was noted to be reduced from 100 to 53% and TUNEL (apoptosis) staining was noted to be increased from 21 to 36 cells (Fig 6.2). The data is in keeping with reduced proliferation and no change in apoptosis as noted with the PET data. The small lymph nodes were not amenable to biopsy.

6.13.2.2 Patient 2

.Patient 2 is a 41 year old woman with a right-sided 70mm Grade 2 invasive ductal carcinoma ER-/PR-/HER2+ invasive ductal carcinoma, who had no involved lymph nodes. The tumour was also noted to have inflammatory components on pathological diagnosis. With PET [¹⁸F]ICMT-11 imaging(Fig 6.3A), uptake was not noted in the post-treatment scan, and histogram analysis showing a similar trend. The histogram analysis also suggests a necrotic response to chemotherapy (Fig 6.3B) while immunohistochemistry correlated with the imaging data with no change in Ki67 or TUNEL noted following chemotherapy (Fig 6.4). In addition, after 3 cycles of chemotherapy, the patient was noted to have stable disease on ultrasound. However, a change in treatment to docetaxel and Herceptin for the next 3 cycles resulted in complete pathological response in the surgical specimen suggesting that early optimisation of chemotherapy would have been useful with a molecular imaging biomarker.

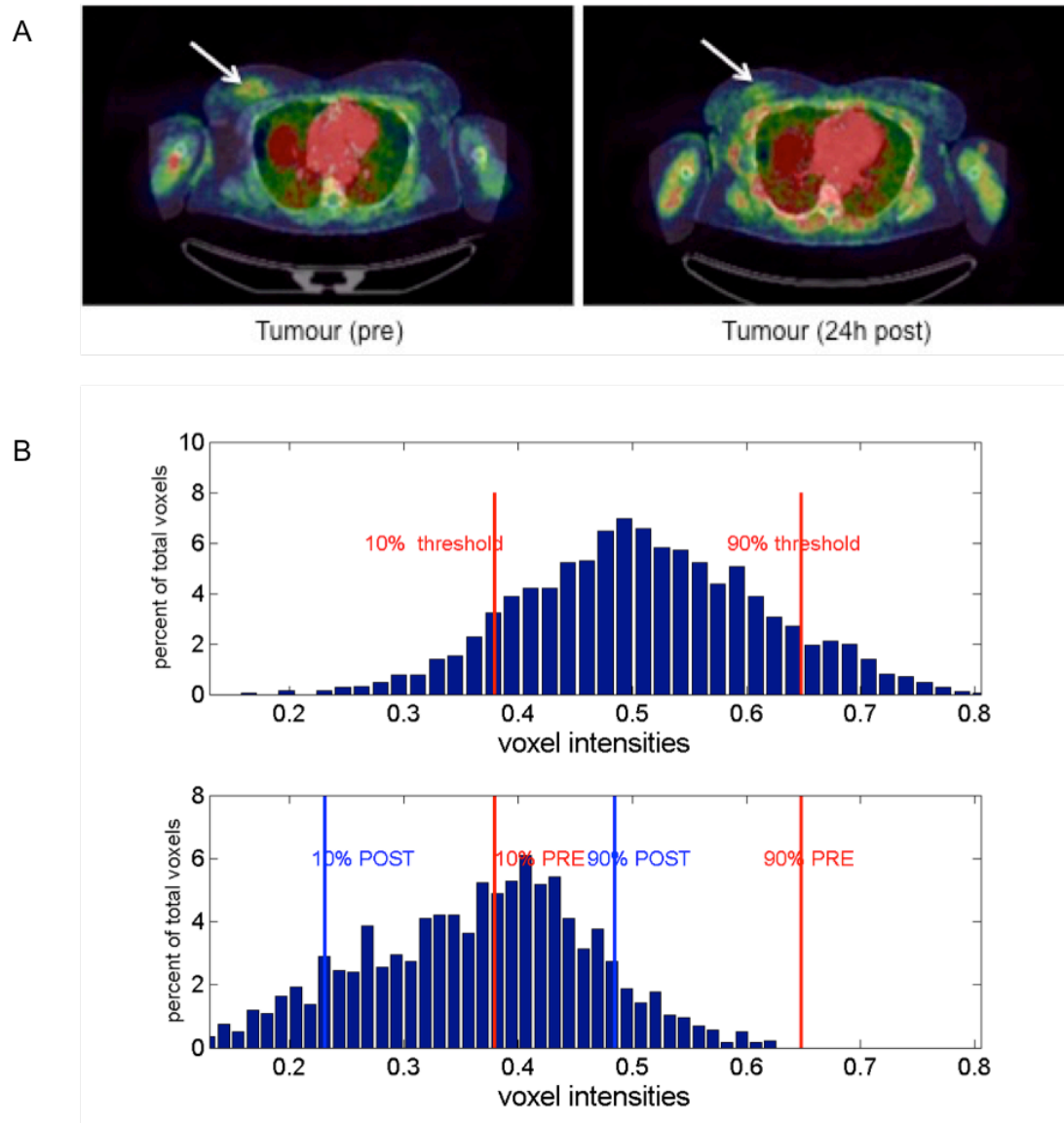


Figure 6-3: Patient 2.

Patient with a 70mm Grade 2 IDC ER-/PR-/HER2+ tumour with inflammatory changes. PET scans were conducted pre-chemotherapy and 24h post chemotherapy (A). Voxel analysis by histogram showed a decrease in intensity of uptake of radiotracer 24h post-chemotherapy.

Table 6-3: Patient 2 parameters.

Tumour and lymph node (LN) SUV (standardized uptake value) have been assessed at 60min. Abbreviations TBR, Tumour to breast ratio; TMR, tumour to muscle ratio; av, average; max, maximum. *denotes increase in parameter consistent with change in apoptosis.

	Pre-chemotherapy	Post-chemotherapy
SUV_{av}	0.44	0.40
SUV_{max}	1.04	0.96
TBR_{max}	1.82*	1.96*
TMR_{max}	1.65	1.23

Immunohistochemistry

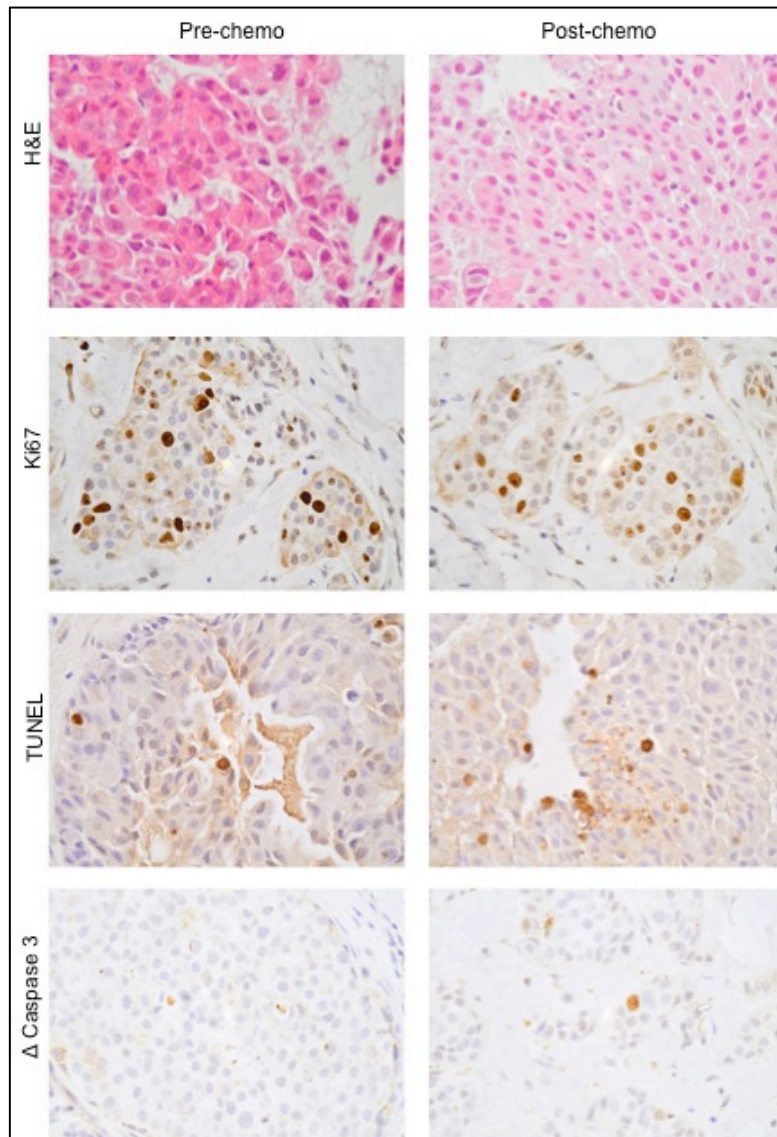


Figure 6-4: Patient 2 immunochemistry.

Immunohistochemistry with Ki67, TUNEL and cleaved caspase 3. Ki67 score was reduced from 29% to 22% following chemotherapy. TUNEL score remained unchanged from 38 to 35. n=3 tumour sections with 5-10 random field of view (FOV) per section. 400x magnification

On immunohistochemical analysis, the Ki67 score was slightly reduced following chemotherapy and similar to the imaging data, no change in TUNEL staining was noted.

6.13.2.3 Patient 3

Patient 3 is a 64 year old woman with a right-sided 50mm Grade 3 invasive ductal carcinoma ER-PR-HER2- (triple negative) with ipsilateral lymph node involvement. The tumour exhibited a central area of necrosis (Figure 6.5). SUV values were not increased in the tumour 48h following chemotherapy. Similar to patient 1, increased uptake was noted in the lymph nodes following chemotherapy SUV_{av} 0.41 (pre) and 0.47(post) and SUV_{max} 0.85 (pre) and 1.31 (post) (Table 6.4). As this histological subtype is sensitive to chemotherapy, the interim ultrasound following 3 cycles of chemotherapy was noted to show a partial response to chemotherapy.

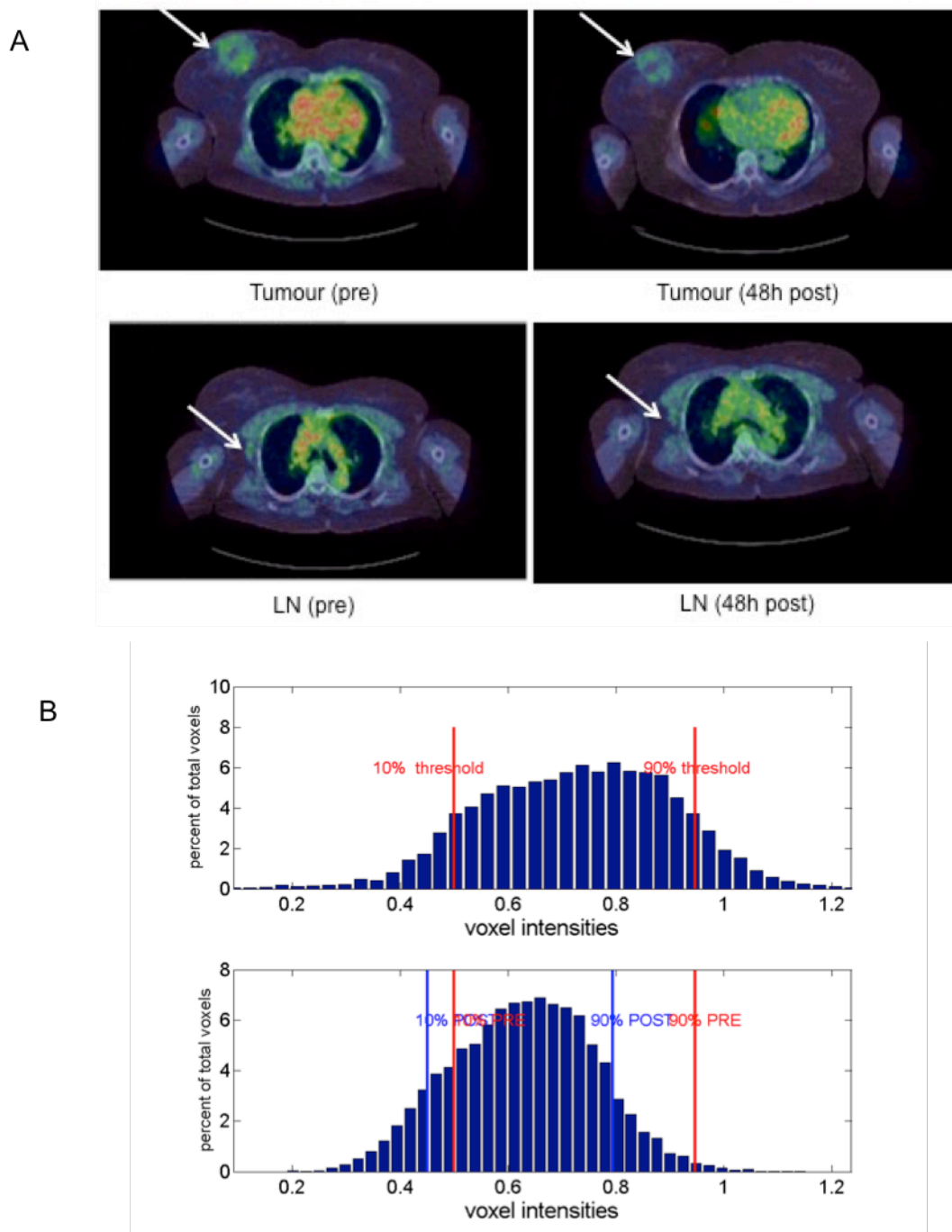


Figure 6-5: Patient 3.

Patient with a 50mm Grade 3 invasive ductal carcinoma, ER-, PR-, HER2-PET scans were conducted pre-chemotherapy and 48h post chemotherapy (A). Voxel analysis by histogram showed a decrease in voxel intensity following chemotherapy (B)

Table 6-4: Patient 3 parameters.

Tumour and lymph node (LN) SUV (standardized uptake value) have been assessed at 60min. Abbreviations TBR, Tumour to breast ratio; TMR, tumour to muscle ratio; av, average; max, maximum. *denotes increase in parameter consistent with change in apoptosis.

	Pre-chemotherapy	Post-chemotherapy
Tumour SUV_{av}	0.6	0.53
Tumour SUV_{max}	1.4	1.34
LN SUV_{av}	0.41*	0.47*
LN SUV_{max}	0.85*	1.31*
TBR_{max}	1.12*	2.12*
TMR_{max}	1.75	1.31

Immunohistochemistry

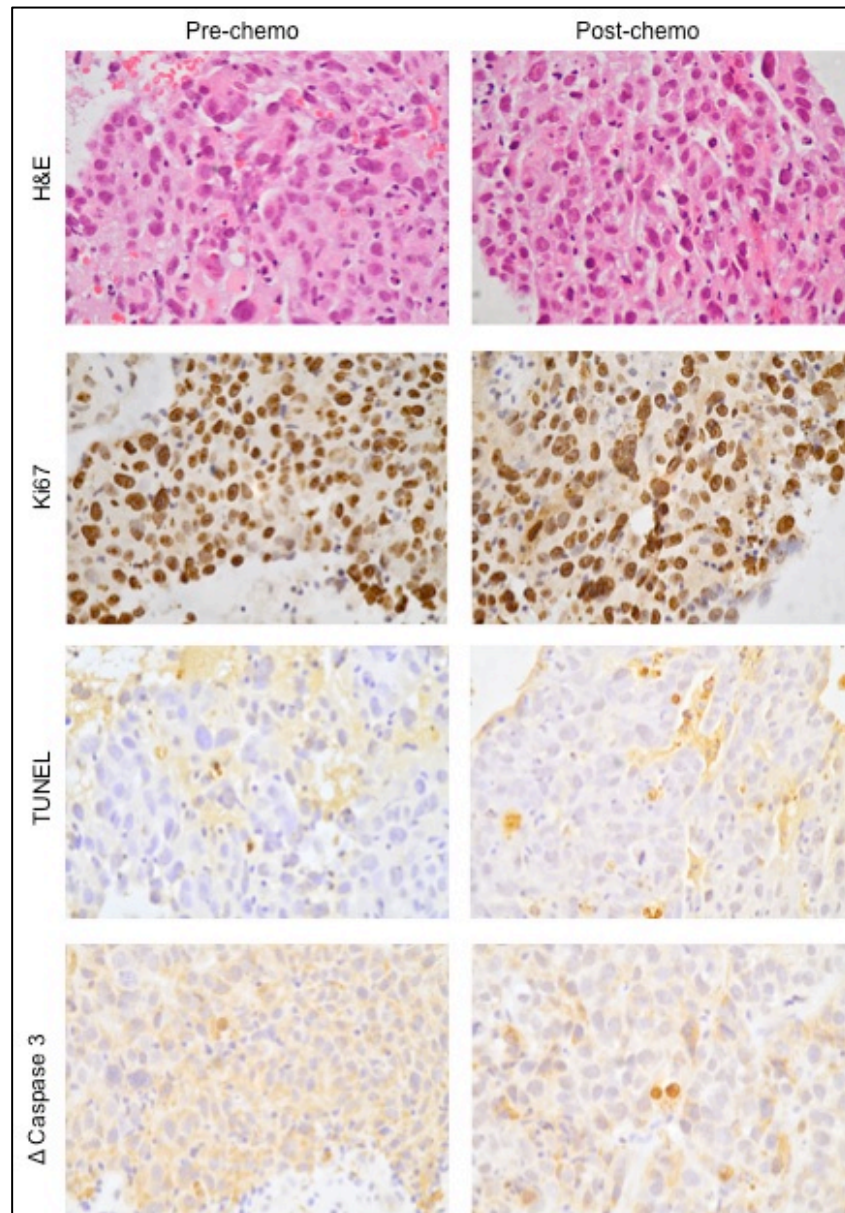


Figure 6-6: Patient 3 immunohistochemistry.

Immunohistochemistry with Ki67, TUNEL and cleaved caspase 3. Ki67 score was unchanged at 100% following chemotherapy. TUNEL score increased from 34 to 64. $n=3$ tumour sections with 5-10 random field of view (FOV) per section. 400x magnification

Although uptake on imaging was not consistent with apoptosis, immunohistochemistry showed a decrease in proliferation (Ki67) and an increase in the apoptosis marker TUNEL (Fig 6.6).

In summary, patients were imaged with [^{18}F]ICMT-11 24-48h following FEC chemotherapy. Uptake of the radiotracer was not noted within the primary tumour but lymph nodes exhibited increased uptake (as determined by SUV) with the apoptosis radiotracer, SUV in the lymph nodes; SUV_{av} 0.39 ± 0.02 (pre), SUV_{av} 0.45 ± 0.03 (post), and SUV_{max} 0.87 ± 0.02 (pre), 1.22 ± 0.12 (post). Although the main breast tumours were biopsied for immunohistochemical assessment, the lymph nodes could not be accessed, therefore a correlation of increased apoptosis could not be assessed.

6.13.3 Patients studied at 1-2 weeks after initiating chemotherapy

6.13.3.1 Patient 4

Patient 4 is a 54 year old woman with a left sided Grade 2 70mm invasive ductal carcinoma ER+,PR+, HER2- and underwent PET imaging with [^{18}F]ICMT-11 prior to and 1 week post chemotherapy (Fig 6.7). Uptake following chemotherapy was reduced similar to the earlier time points of 24-48h (Table 6.5). Response to chemotherapy was also favourable with partial response noted on ultrasound after 3 cycles of chemotherapy.

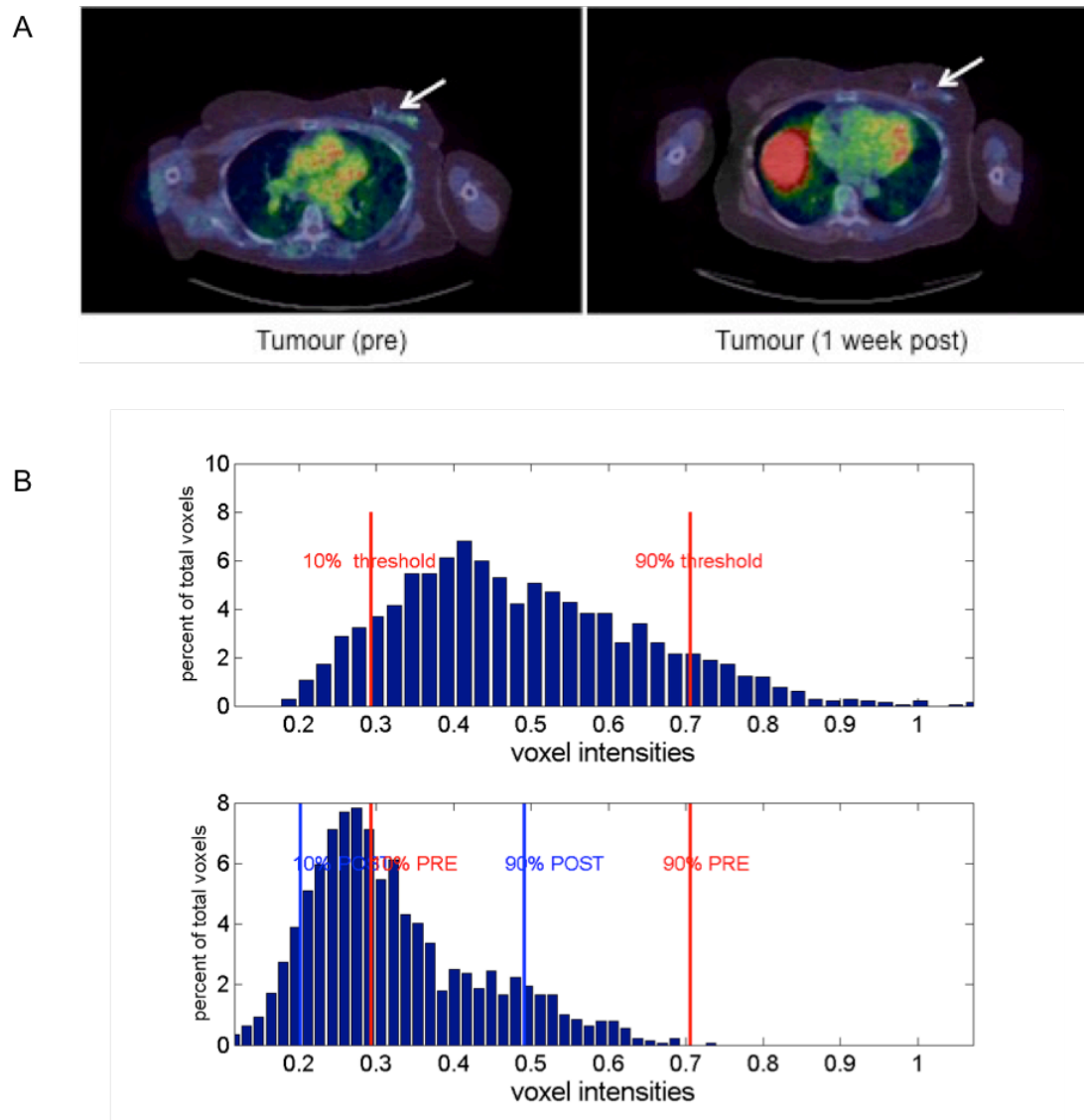


Figure 6-7: Patient 4.

70mm Grade 2 invasive ductal carcinoma ER+,PR+, HER2-. Patient had a PET scan prior to and 1 week post chemotherapy to assess apoptosis at this time point (A). Voxel analysis by histogram shows a decrease in voxel intensity following chemotherapy (B)

Table 6-5: Patient 4 parameters.

Tumour SUV (standardized uptake value) have been assessed at 60mins. Abbreviations TBR, Tumour to breast ratio; TMR, tumour to muscle ratio; av, average; max, maximum.

*denotes increase in parameter consistent with change in apoptosis.

	Pre-chemotherapy	Post-chemotherapy
SUV_{av}	0.43	0.19
SUV_{max}	1.01	0.77
TBR_{max}	2.15	2.14
TMR_{max}	1.42*	1.67*

Immunohistochemistry

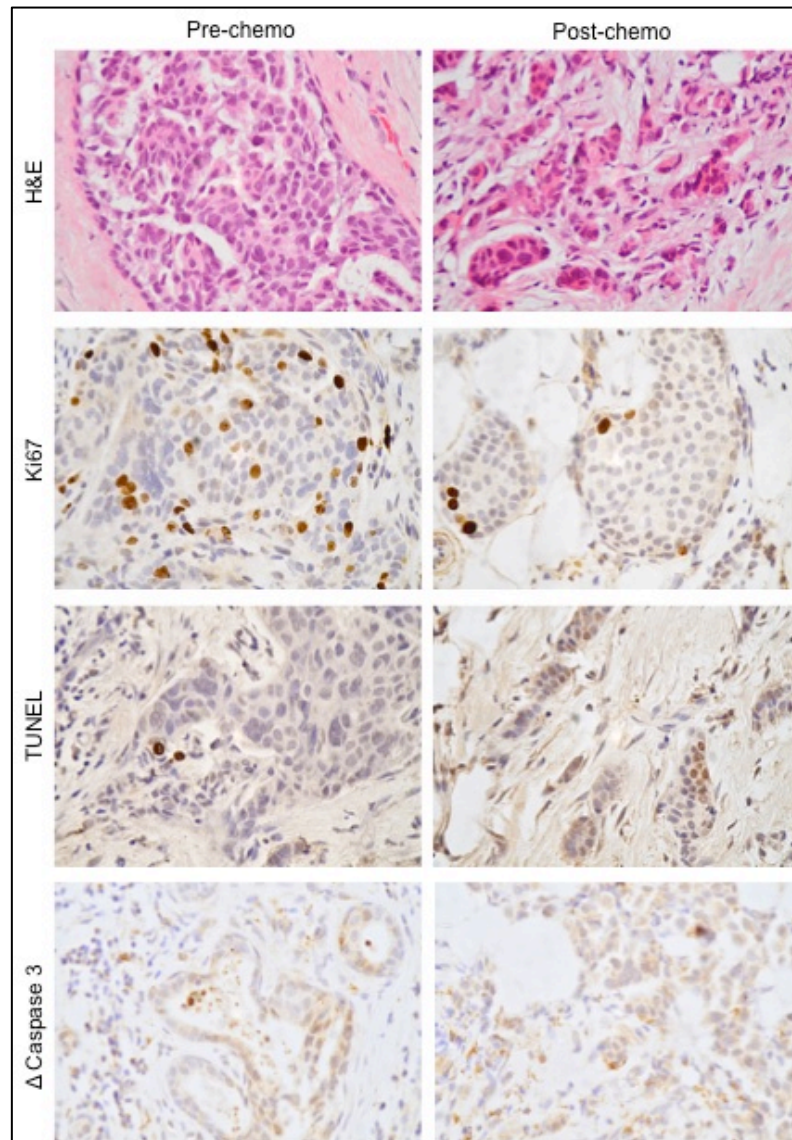


Figure 6-8: Patient 4 immunohistochemistry.

Immunohistochemistry with Ki67, TUNEL and cleaved caspase 3. Ki67 score was decreased from 30% to 7% following chemotherapy. TUNEL score unchanged from 33 to 24. $n=3$ tumour sections with 5-10 random field of view (FOV) per section. 400x magnification

Immunohistochemistry of the tumour following chemotherapy revealed a decrease in proliferation while TUNEL analysis was unchanged (Fig 6.8).

6.13.3.2 Patient 5

Patient 5 is a 56 year old woman with a left sided 30mm Grade 3 invasive ductal carcinoma (ER+,PR+, HER2-) with ipsilateral lymph node involvement. The patient was imaged with [^{18}F]ICMT-11 prior to and 2 weeks post FEC chemotherapy (Fig 6.9A). Uptake with the apoptosis PET tracer was noted to be increased following chemotherapy SUV_{av} 0.48(pre), 0.57 (post); SUV_{max} 1.08(pre), 1.16(post) (Table 6.6). The histogram analysis of voxel intensity also demonstrated an increase in intensity following chemotherapy consistent with apoptosis(Fig 6.7B). The patient also had a partial response to chemotherapy as assessed by ultrasound during the interim point evaluation.

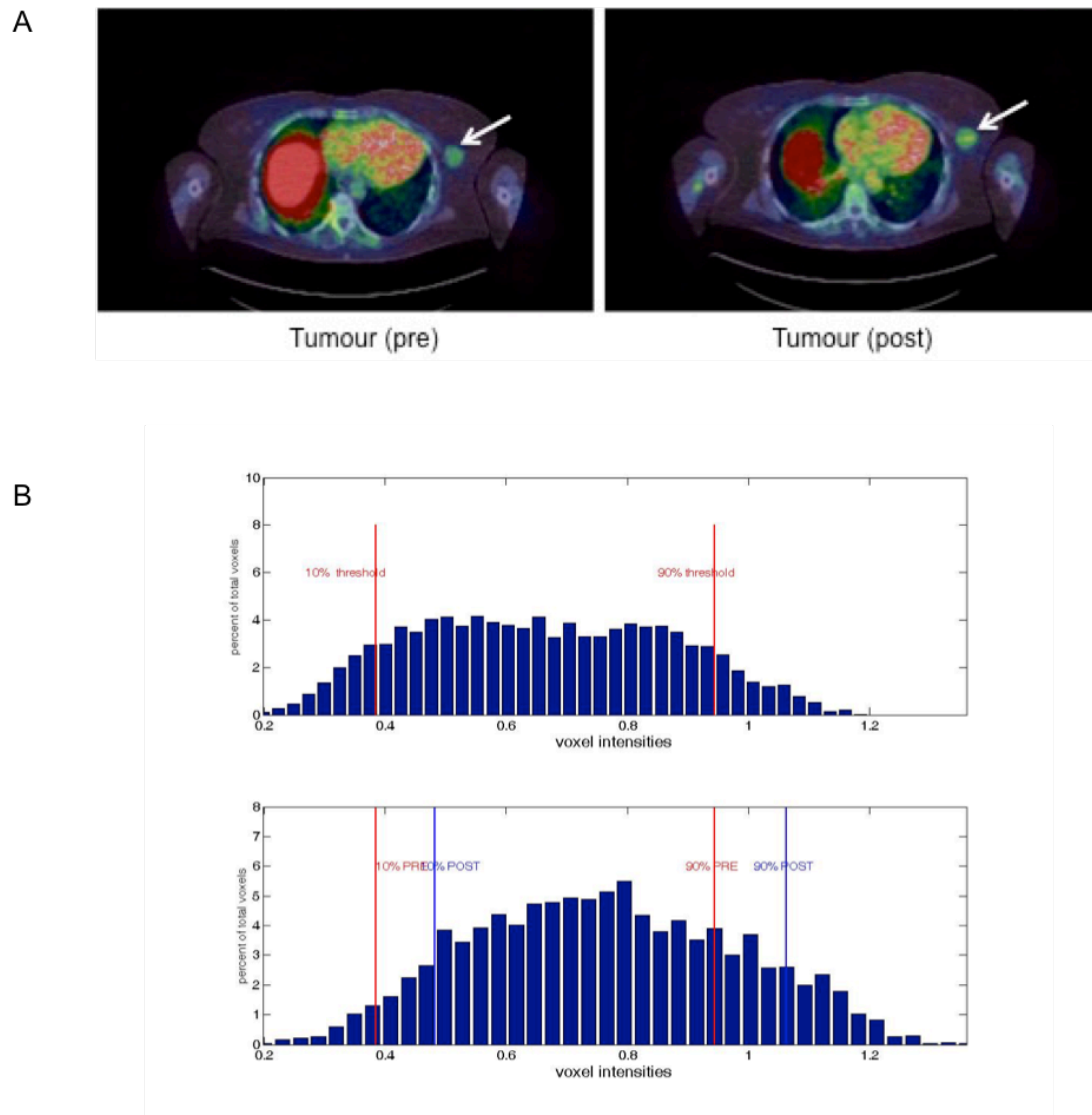


Figure 6-9: Patient 5.

Patient with Grade 3 30mm invasive ductal carcinoma ER+,PR+, HER2-. Patient underwent PETCT prior to chemotherapy and 2 weeks post-chemotherapy (A). Voxel analysis by histogram reveals increase in voxel intensity post-chemotherapy consistent with increased apoptosis post-chemotherapy.

Table 6-6: Patient 5 parameters.

Tumour SUV (standardized uptake value) have been assessed at 60min. Abbreviations TBR, Tumour to breast ratio; TMR, tumour to muscle ratio; av, average; max, maximum. *denotes increase in parameter consistent with change in apoptosis.

	Pre-chemotherapy	Post-chemotherapy
SUV_{av}	0.48*	0.57*
SUV_{max}	1.08*	1.16*
TBR_{max}	1.29*	2.04*
TMR_{max}	1.93*	2.04*

Immunohistochemistry

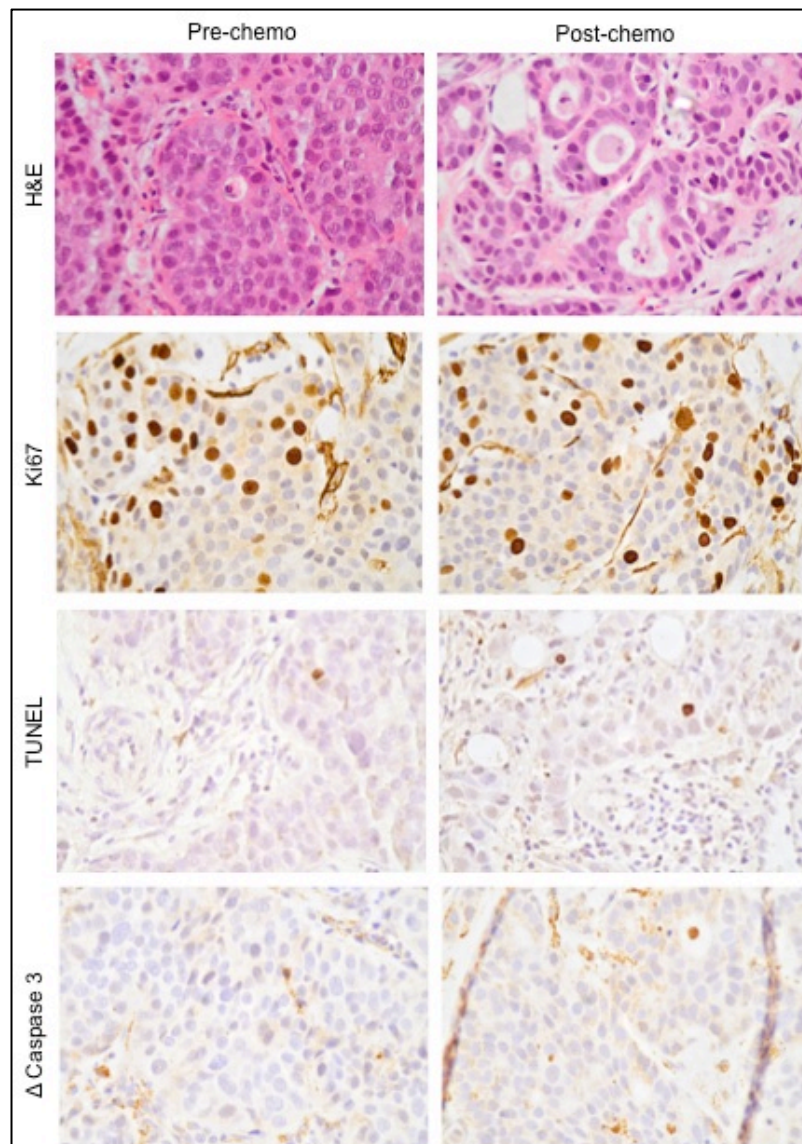


Figure 6-10: Patient 5 immunohistochemistry.

Immunohistochemistry with Ki67, TUNEL and cleaved caspase 3. Ki67 score was unchanged from 26% from 28% following chemotherapy. TUNEL score was however increased from 27 to 77. $n=3$ tumour sections with 5-10 random field of view (FOV) per section. 400x magnification

In keeping with the imaging findings, the TUNEL score was increased following chemotherapy suggestive of correlation with apoptosis (Fig 6.10). However, the proliferation index was unchanged on immunohistochemical analysis.

6.13.3.3 Patient 6

Patient 6 is 43 year old woman with a 25mm Grade 2 invasive ductal carcinoma ER+, PR+, HER2- who underwent imaging prior to chemotherapy and 2 weeks following chemotherapy (Fig 6.11A). Uptake of the apoptosis tracer was noted to increase following chemotherapy SUV_{av} 0.54(pre) and 0.63(post), SUV_{max} 0.87 (pre), 1.37 (post)(Table 6.7). Histogram analysis demonstrated a right shift of voxel intensity in keeping with apoptosis (Fig 6.11B). There was a partial response observed on ultrasound after 3 cycles of chemotherapy. High background uptake is noted within the liver.

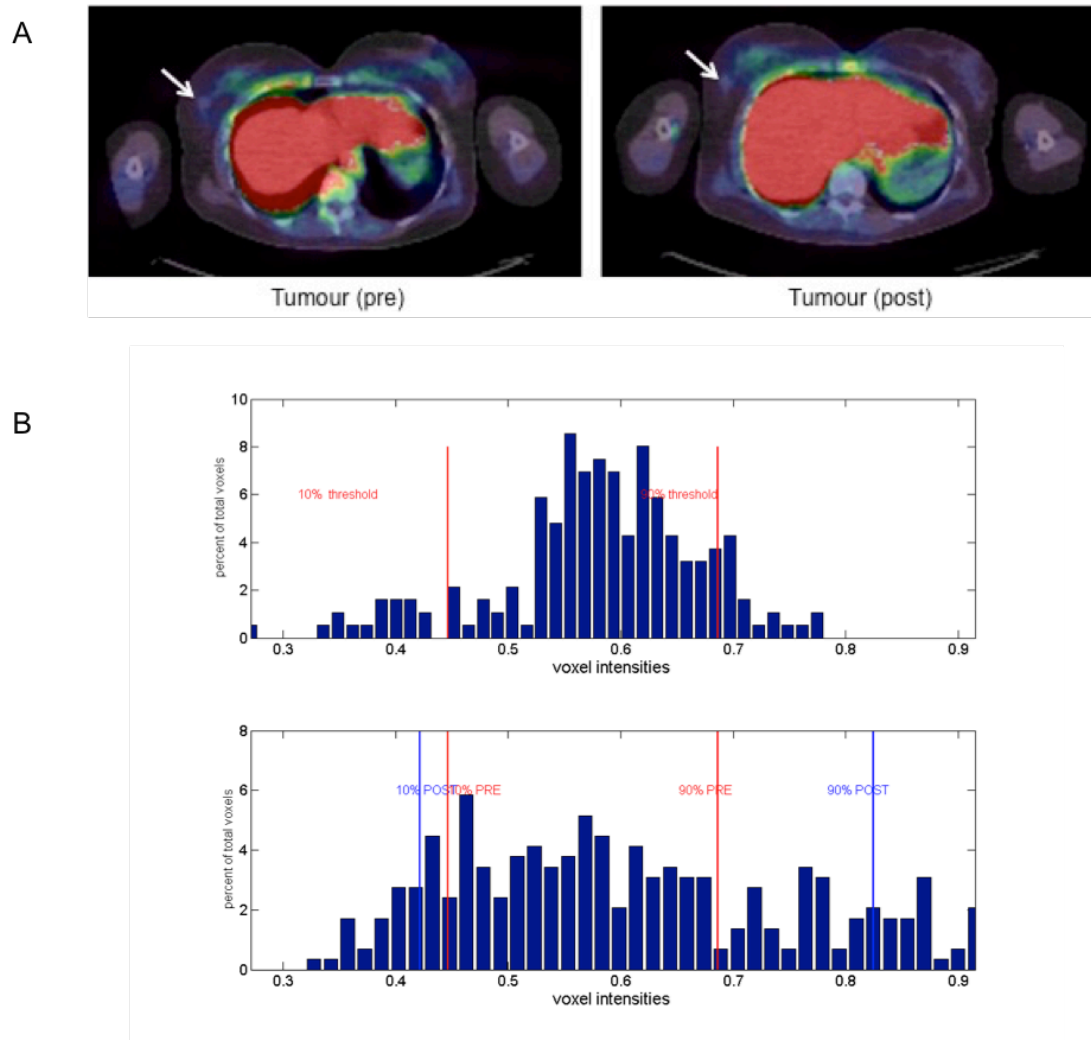


Figure 6-11: Patient 6.

Patient with a 25mm Grade 2 invasive ductal carcinoma, ER+, PR+, HER2-ve tumour. Patient underwent PETCT pre-chemotherapy and 2 weeks post-chemotherapy (A). Voxel analysis by histogram reveals increase in voxel intensity post-chemotherapy consistent with increased apoptosis post=chemotherapy (B).

Table 6-7: Patient 6 parameters.

Tumour SUV (standardized uptake value) have been assessed at 60min. Abbreviations TBR, Tumour to breast ratio; TMR, tumour to muscle ratio; av, average; max, maximum. *denotes increase in parameter consistent with change in apoptosis.

	Pre-chemotherapy	Post-chemotherapy
SUV_{av}	0.54*	0.63*
SUV_{max}	0.87*	1.37*
TBR_{max}	1.72*	3.0*
TMR_{max}	1.72*	2.12*

Immunohistochemistry

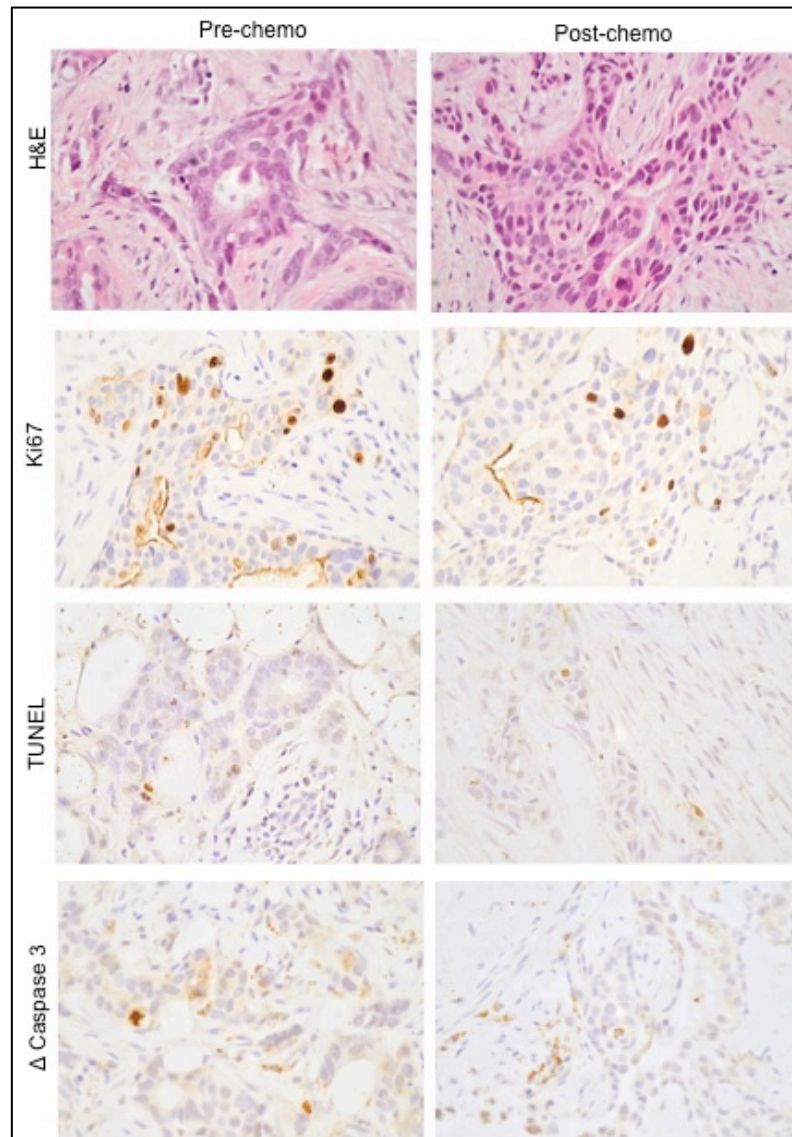


Figure 6-12: Patient 6 immunohistochemistry.

Immunohistochemistry with Ki67, TUNEL and cleaved caspase 3. Ki67 score was reduced from 27% from 12% following chemotherapy. TUNEL score was however increased from 7 to 10. $n=3$ tumour sections with 5-10 random field of view (FOV) per section. 400x magnification

On immunohistochemical findings for this patient, the TUNEL score was also increased in keeping with apoptosis and in addition the proliferation index was also reduced following chemotherapy (Fig 6.12).

6.13.3.4 Patient 7

Patient 7 is a 67 year old woman with a right-sided 32mm Grade 3 invasive ductal carcinoma ER+,PR+,HER2- with ipsilateral lymph node involvement. The patient was imaged as baseline and 2 weeks post chemotherapy with FEC (Fig 6.13A). Both the tumour and lymph node did not exhibit increased uptake of tracer following chemotherapy. However, a favourable response of partial response was noted on the ultrasound following 3 cycles of chemotherapy.

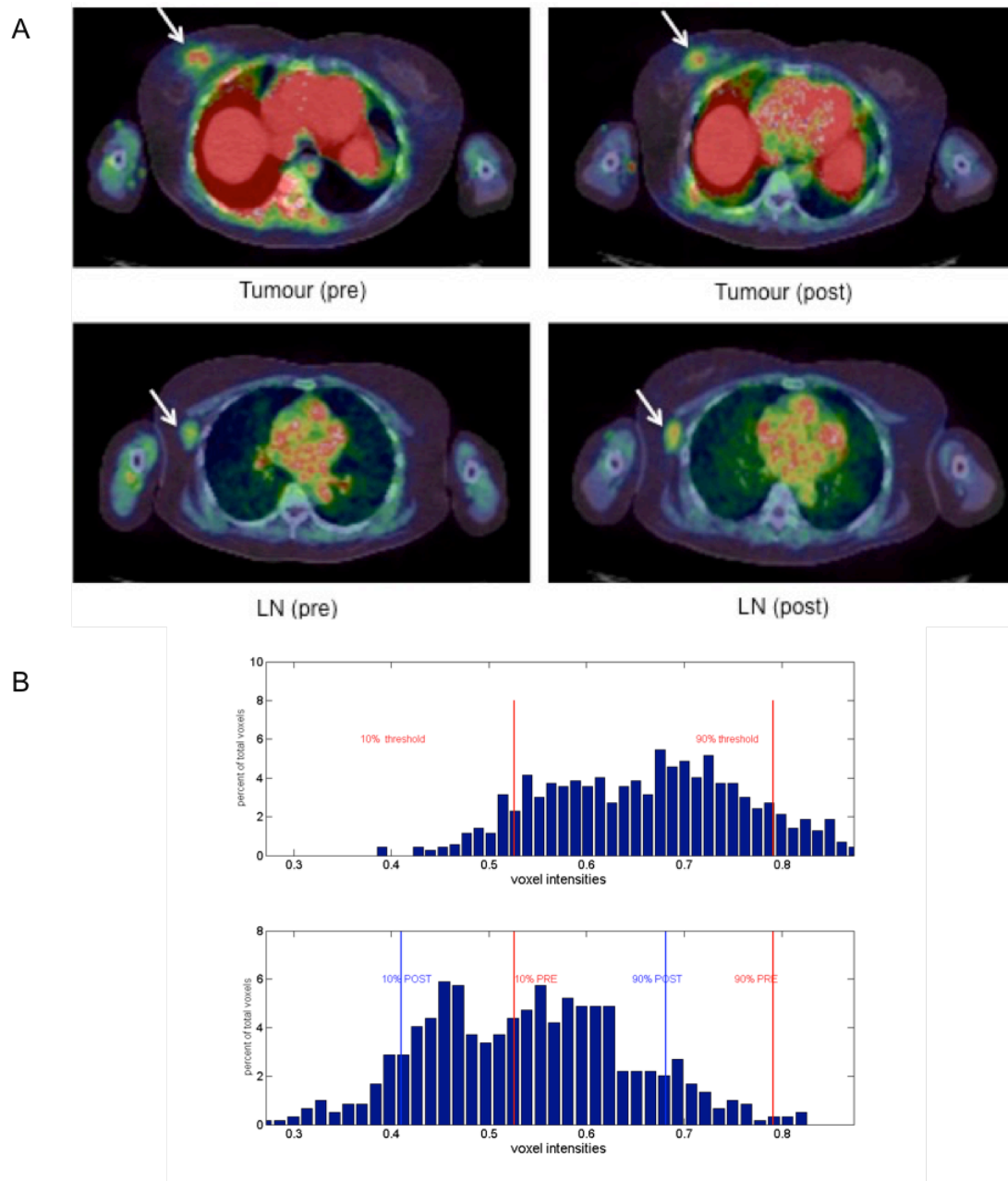


Figure 6-13: Patient 7.

Patient with 32mm Grade 3 invasive ductal carcinoma, ER+, PR+, HER2 –ve. Patient was imaged pre-chemotherapy and 2 weeks post chemotherapy (A). Voxel analysis by histogram reveals a decrease in voxel intensity post-chemotherapy (B)

Table 6-8: Patient 7 parameters.

Tumour SUV (standardized uptake value) have been assessed at 60min. Abbreviations LN; lymph node, TBR, Tumour to breast ratio; TMR, tumour to muscle ratio; av, average; max, maximum.

	Pre-chemotherapy	Post-chemotherapy
Tumour SUV_{av}	1.0	0.68
Tumour SUV_{max}	1.71	1.12
LN SUV_{av}	0.55	0.24
LN SUV_{max}	0.93	0.52
TBR_{max}	6.0	2.50
TMR_{max}	3.14	2.24

Immunohistochemistry

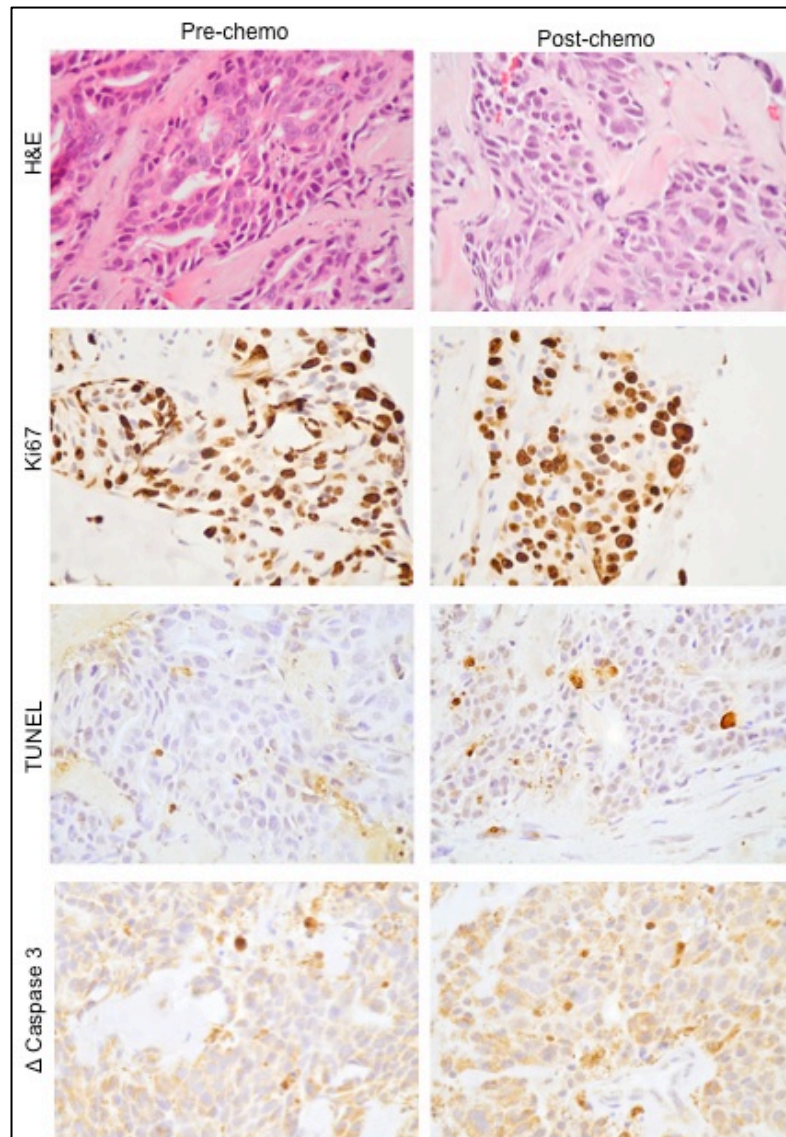


Figure 6-14: Patient 7 immunohistochemistry.

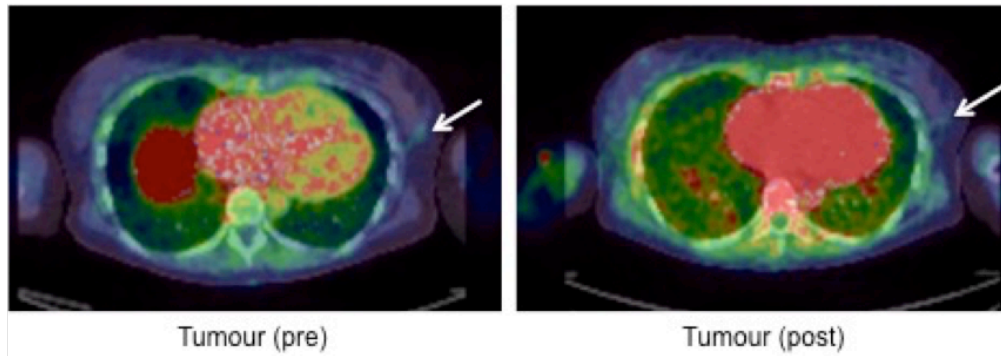
Immunohistochemistry with Ki67, TUNEL and cleaved caspase 3. Ki67 score was unchanged from 67% to 70% following chemotherapy. TUNEL score was however increased from 18 to 56. $n=3$ tumour sections with 5-10 random field of view (FOV) per section. 400x magnification.

On immunohistochemical analysis, the proliferation index Ki67 was unchanged while TUNEL staining was increased in keeping with apoptosis but did not correlate to PET tracer uptake findings (Fig 6.14)

6.13.3.5 Patient 8

Patient 8 is a 45 year old woman with a left sided 18mm Grade 3 invasive ductal carcinoma ER-,PR-,HER2- with ipsilateral axillary lymphadenopathy. This patient was imaged at baseline and 2 weeks post chemotherapy with FEC (Fig 6.15A). Following chemotherapy, there was a decrease in uptake of the tracer. The patient responded favourably to chemotherapy with partial response noted after 3 cycles of chemotherapy.

A



B

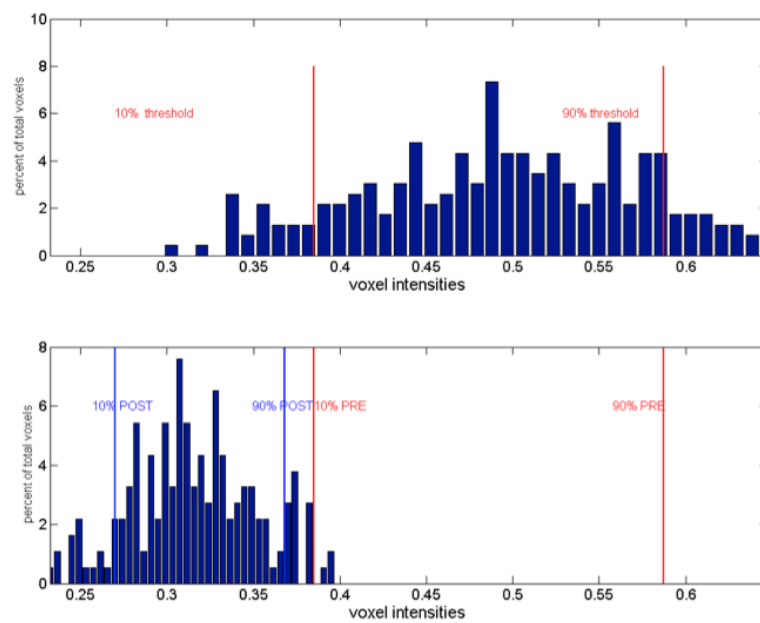


Figure 6-15: Patient 8.

Patient with 18mm Grade 3 invasive ductal carcinoma, ER-, PR-, HER2 –ve. Patient was imaged pre-chemotherapy and 2 weeks post chemotherapy (A). Voxel analysis by histogram reveals a decrease in voxel intensity post-chemotherapy (B)

Table 6-9: Patient 8 parameters.

Tumour SUV (standardized uptake value) have been assessed at 60min. Abbreviations: TBR, Tumour to breast ratio; TMR, tumour to muscle ratio; av, average; max, maximum

	Pre-chemotherapy	Post-chemotherapy
SUV_{av}	0.42	0.39
SUV_{max}	0.65	0.56
TBR_{max}	1.40	1.03
TMR_{max}	1.47	0.88

Immunohistochemistry

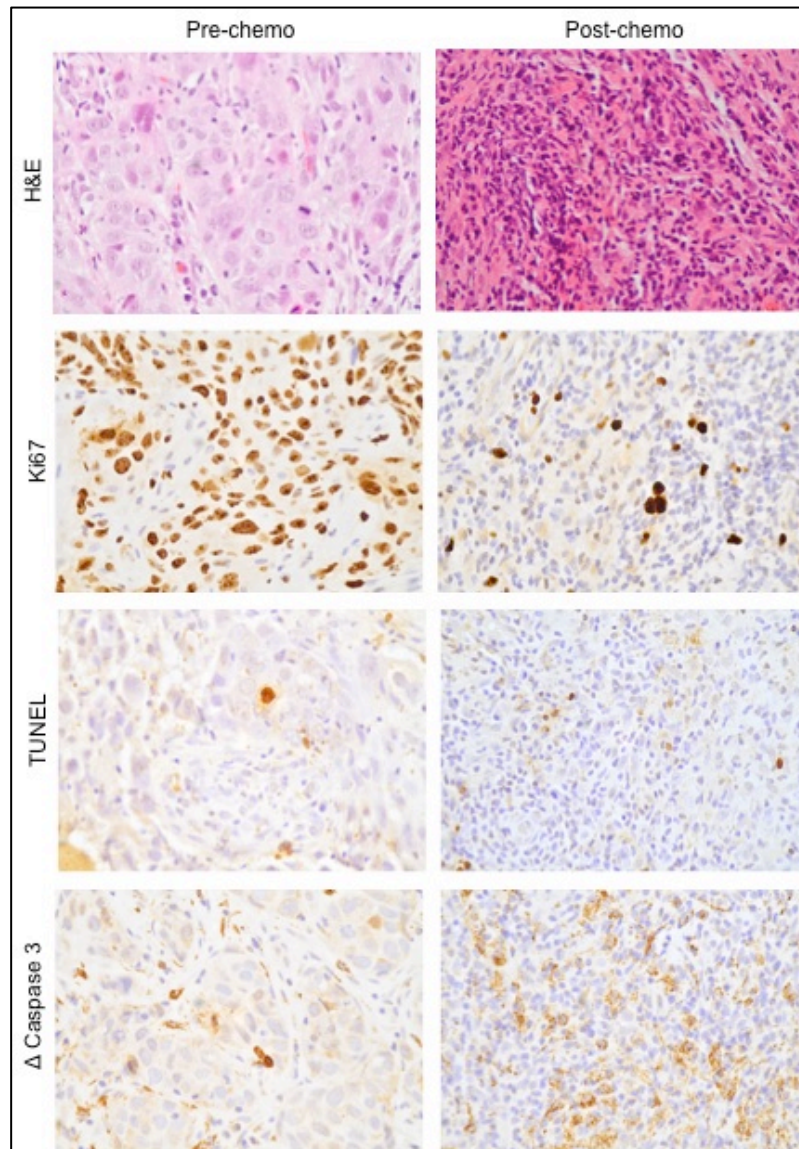


Figure 6-16: Patient 8 immunohistochemistry.

Immunohistochemistry with Ki67, TUNEL and cleaved caspase 3. Ki67 score was 100% in tumour sample pre-chemotherapy and no tumour was seen in the post-chemotherapy sample. TUNEL score on pre-chemotherapy sample was 18 and no tumour was seen in the post treatment sample. $n=3$ tumour sections with 5-10 random field of view (FOV) per section. 400x magnification

On immunohistochemistry, the patient's tumour was not visible on the sample obtained from biopsy suggesting either a very favourable response to chemotherapy or a sampling error (Fig 6.16).

In summary, the cohort of patients who were imaged between 1-2 weeks following chemotherapy, exhibited a mixed response. 2 patients had high uptake of [^{18}F]ICMT-11 while 3 patients had no change. However, tumour to breast ratio (TBR) and tumour to muscle ratio (TMR) were increased 1.50 ± 0.22 (pre), 2.52 ± 0.48 (post), and 1.82 ± 0.10 (pre), 2.08 ± 0.04 (post), respectively. It is difficult to ascertain why there is variation in the data as the data is not sufficient to determine whether it is related to histological subtype or size of the tumour. Due to limitations with immunohistochemistry, it was not possible to correlate the imaging data with apoptosis on immunohistochemistry.

6.13.4 Discussion

Neoadjuvant chemotherapy in clinical breast cancer can offer several advantages such as downstaging large tumours and therefore improve the rate of breast conserving surgery, in addition to reducing micrometastatic spread. Certainly, chemotherapy regimes leading to complete pathological response in surgical specimens are associated with long-term disease survival. Neoadjuvant chemotherapy provides an opportunity to assess response to treatment *in vivo* prior to full surgical resection. Non-invasive imaging of treatment response is currently identified from ultrasound, mammograms and magnetic resonance imaging prior to surgery. However, molecular imaging techniques of apoptosis by PET following chemotherapy could rapidly provide earlier response assessment than current modalities and could allow for treatment modification in patients not responding well to chemotherapy. PET studies of apoptosis could provide a surrogate marker of clinical response in those tumours that show early responses to treatment.

Apoptosis following chemotherapy is known to occur at an early time point and can occur within 24 hours of starting chemotherapy. This is also evident from pre-clinical models [114-116]. However, in clinical studies, apoptosis assessment by immunohistochemistry analysis varies widely from 24 hours following cancer treatment such as chemo or radiotherapy up to 6 weeks following chemotherapy. Radiotracers used for imaging apoptosis in clinical studies include [^{18}F]Annexin-V and [^{18}F]ML-10 and have shown changes in apoptosis following chemotherapy, radiotherapy and chemoradiotherapy in various tumour types. These were clinically relevant as increased uptake was

associated with response to treatment. Although [^{18}F]ML-10 has been noted to be safely used clinically with favourable dosimetry and safety profile, [^{18}F]Annexin-V has shown low specificity of apoptosis over necrosis, problems with biodistribution and slow clearance from targeted tissues [125].

We have developed an apoptosis tracer [^{18}F]ICMT-11 with subnanomolar affinity to the executioner cleaved caspase 3. Dosimetry studies have shown it to be safe and tolerated well[137]. In this study, as part of a clinical trial in neoadjuvant chemotherapy, 8 patients have undergone PET imaging prior to chemotherapy and between 24h and 2 weeks post chemotherapy. The breast tumours were representative of the pathological spectrum including ER,PR+ve, Triple negative and HER2+. Patients were initially imaged pre-chemotherapy with FEC and 24h post chemotherapy with a post-chemotherapy biopsy obtained within a few hours of the post-chemotherapy PET scan.

In our study, 3 patients initially imaged at 24hours following chemotherapy did not express increased uptake of [^{18}F]ICMT-11 within the tumour. This is in contrast to the pre-clinical data where apoptosis was noted within 24h and sustained up to 96h following treatment. The histological subtypes imaged included ER+,PR+ and HER2+ tumours. There was, however, increased uptake noted in the lymph nodes at 24h following chemotherapy. The lymph nodes being much smaller than original tumour, probably have a higher rate of apoptosis than the tumour which was better visualised on PET imaging. The imaging could not be correlated to immunohistochemistry as the smaller lymph nodes were not accessible to biopsy.

As breast cancer is not as rapidly proliferating a tumour as lymphoma for example, it was hypothesised that apoptosis may be occurring at a later time point and therefore patients were then imaged with [^{18}F]ICMT-11 at 1-2 weeks following chemotherapy. 2 patients with ER+,PR+ tumours demonstrated an increase in uptake on PET imaging 2 weeks after chemotherapy. This was also correlated to immunohistochemistry with increased staining on the TUNEL assay.

A further factor of consideration is the voxel analysis of histogram data. A left shift of voxels on the histogram is associated with necrosis while a right shift or increased voxel intensity is associated with apoptosis. As TUNEL staining is indicative of cell death, be it apoptosis or necrosis, a more specific marker of apoptosis such as cleaved caspase 3 is necessary to confirm the presence of apoptosis. Although all patients but one (Patient 2, note, inflammatory component of breast cancer) had response to chemotherapy after 3 cycles, the histogram analysis suggests that some had a necrotic response to chemotherapy rather than apoptotic. Optimisation of immunohistochemical stains of TUNEL and caspase are required before confirmation of the nature of response to chemotherapy and therefore clinical response.

As the study is currently on going, some factors need consideration. The time point for imaging apoptosis in breast cancer is still being evaluated. At the earlier time points of 24-48h, the smaller lymph nodes exhibit increased uptake compared to the tumour. It is likely that apoptosis is occurring in the tumour itself at a smaller scale than is visualised with the apoptosis radiotracer. At 2 weeks, some favourable responses were observed with

increased uptake noted in the tumour following chemotherapy, however, further studies at this time point need to be continued. Further, the correlation of imaging to immunohistochemistry of biopsied samples needs further optimisation. Although we used TUNEL staining (immunostaining of DNA fragments) to identify apoptotic cells, the gold standard for correlation to apoptosis would be staining of cleaved caspase-3. We are currently trying to optimise this. Breast tumours are also difficult to delineate well on CT, but tumour outlining for the PET scans pre and post chemotherapy is maintained unless there has been a very good response to chemotherapy where tumour is difficult to outline, as has been the case for one patient (Patient 8).

In summary, molecular imaging of apoptosis by PET along with in vivo biopsies provides a unique opportunity to assess early responses to chemotherapy. This would allow early assessment of chemotherapy or other cancer therapies and would allow tailoring of treatment especially in cases of poor responders to treatment. Additionally, PET imaging of apoptosis could be utilised as a surrogate marker for clinical outcomes if apoptosis can be correlated to clinical responses. As the study is still on going, it will provide further information on optimal time points for imaging and correlation to immunohistochemical analysis of the tumour tissue. In addition, other clinical trials of apoptosis imaging in other tumours are also on going in lung cancer and lymphoma to determine time points for imaging apoptosis as well as determine the clinical relevance of such imaging.

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Posters and Publications

Publications

1. Imaging as a pharmacodynamic and response biomarker in cancer. Clinical and Translational Imaging. February 2014. 2(1): p13-31. *Shairoz Merchant, Timothy H Witney, Eric O Aboagye.*
2. Synthesis and pre-clinical evaluation of a [^{18}F]fluoromethyl-Tanaproget derivative for imaging of progesterone receptor expression. RSC Advances June 2016 (Issue 62). *Shairoz Merchant, Louis Allott, Laurence Carroll, Vickram Tittrea, Steven Kealey, Timothy H. Witney, Philip W. Miller, Graham Smith and Eric O. Aboagye*

Poster presentations

1. Development of a pre-clinical Progesterone Receptor (PR) PET radiotracer for breast cancer imaging
Shairoz Merchant, Timothy Witney, Laurence Carroll, Louis Allott, Graham Smith, Quang-De Nguyen, Eric Aboagye.
Shortlisted for the **British Association of Cancer Research (BACR)/Gordon Hamilton-Fairley Young Investigator Award**
2. Evaluation of apoptosis in breast cancer using the novel PET probe [^{18}F]ICMT-11 in patients treated with neoadjuvant FEC chemotherapy: Initial assessment of optimum imaging time and relation to caspase-3 immunostaining
Shairoz Merchant, Eric O Aboagye, Adrian Lim, Kasia Kozlowski, Naina Patel, Jennifer Steel, Susan Cleator, Sami Shousha, Vidhya Varghese, Raoul C Coombes, and Laura Kenny
Awarded the AACR Scholar-in-Training Award – Supported by Susan G. Komen®