Molecular Imaging as a Tool for Predicting and Monitoring Response of Breast Cancer to Trastuzumab (Herceptin®)

By

Kristin McLarty

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
University of Toronto

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ABSTRACT

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Kristin McLarty
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University of Toronto

The human epidermal growth factor receptor 2 (HER2) is overexpressed in 20% of breast cancers (BCs) and confers an aggressive tumour phenotype with a poor prognosis. Trastuzumab (Herceptin®) is a humanized IgG\textsubscript{1} monoclonal antibody (mAb) approved for treatment of HER2-positive breast cancer (BC), however many eligible patients do not respond. The hypothesis was that molecular imaging strategies that probe: i) the expression of HER2; ii) one of the mechanisms of action of trastuzumab or iii) evaluate the viability of tumour cells by their glucose utilization would be useful in predicting and monitoring the response of BC to treatment with trastuzumab. The relationship between tumour HER2 density, uptake of $^{111}$In-DTPA-trastuzumab and response to trastuzumab was evaluated by gamma camera imaging, biodistribution studies and monitoring tumour growth in mice implanted with BC xenografts. There was a non-linear relationship between HER2 expression and uptake of this radiopharmaceutical when tumour uptake was corrected for non-specific IgG accumulation and/or circulating blood radioactivity ($r^2=0.87-0.99$). Tumour response corresponded better with the uncorrected tumour uptake of $^{111}$In-DTPA-trastuzumab. HER2 downregulation, a putative mechanism of action of trastuzumab, was noted as decreased tumour uptake on microSPECT/CT of mice bearing MDA-MB-361 xenografts administered $^{111}$In-DTPA-pertuzumab. Tumour uptake of $^{111}$In-DTPA-pertuzumab was reduced by 53% in mice treated for 3 days with trastuzumab (P<0.05) associated with an early molecular response to the drug. Furthermore, tumour uptake of $^{111}$In-DTPA-pertuzumab was reduced by 78% (P<0.001) in mice...
treated for 3 weeks, which corresponded with a reduction in HER2-positive tumour cells, indicating a therapeutic response. The relationship between changes in tumour uptake of $^{18}$F-2-fluoro-2-deoxy-D-glucose ($^{18}$F-FDG) and response to trastuzumab was examined in mice bearing MDA-MB-361 and MDA-MB-231 BC xenografts, with high or very low HER2 expression, treated with trastuzumab. MicroPET imaging and biodistribution studies detected a 43-60% (P<0.03) reduction in tumour uptake of $^{18}$F-FDG in mice with MDA-MB-361 xenografts, treated with trastuzumab compared to PBS-treated controls. In contrast, there was no change in $^{18}$F-FDG uptake in MDA-MB-231 xenografts, that did not respond to trastuzumab. I conclude that molecular imaging is a promising tool for monitoring response of BC to treatment with trastuzumab.
ACKNOWLEDGEMENTS

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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>%ID/g</td>
<td>percent injected dose per gram of tissue</td>
</tr>
<tr>
<td>%ID/pixel</td>
<td>percent injected dose per pixel</td>
</tr>
<tr>
<td>%ID/voxel</td>
<td>percent injected dose per voxel</td>
</tr>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>17-AAG</td>
<td>17-allylaminogeldamycin</td>
</tr>
<tr>
<td>17-DMAG</td>
<td>17-demethoxygeldanamycin</td>
</tr>
<tr>
<td>$^{18}$F-FDG</td>
<td>$[^{18}F]$-fluoro-2-deoxy-D-glucose</td>
</tr>
<tr>
<td>$^{18}$F-FES</td>
<td>$16\alpha$-$[^{18}F]$-fluoro-17$\beta$-estradiol</td>
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<tr>
<td>$^{18}$F-FLT</td>
<td>3'-deoxy-3'$[^{18}F]$-fluorothymidine</td>
</tr>
<tr>
<td>$^{68}$Ga-DCHF</td>
<td>$^{68}$Ga-DOTA conjugated herceptin Fab</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>indium-111</td>
</tr>
<tr>
<td>AC</td>
<td>cyclophosphamide and doxorubicin</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase also known as protein kinase B</td>
</tr>
<tr>
<td>Ang-1</td>
<td>angioptiotin-1</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ASCO</td>
<td>American Society of Clinical Oncology</td>
</tr>
<tr>
<td>BC</td>
<td>breast cancer</td>
</tr>
<tr>
<td>BCIRG 006</td>
<td>the breast cancer international research group trial 006</td>
</tr>
<tr>
<td>BCS</td>
<td>breast conserving surgery</td>
</tr>
<tr>
<td>BGO</td>
<td>bismuth germanate crystal</td>
</tr>
<tr>
<td>$B_{\text{max}}$</td>
<td>amount of antibody required to saturate available receptors (nmol)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSGI</td>
<td>breast specific gamma imaging</td>
</tr>
<tr>
<td>CAP</td>
<td>College of American Pathologists</td>
</tr>
<tr>
<td>CB11</td>
<td>anti-HER2 antibody, clone CB11</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CDR</td>
<td>complimentary determining region</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
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<td>CEF</td>
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<td>centromere 17</td>
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<tr>
<td>CMF</td>
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<tr>
<td>CNB</td>
<td>core needle biopsy</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal carcinoma</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>DAPI</td>
<td>4′-6′-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCE-MRI</td>
<td>dynamic contrast-enhanced magnetic resonance imaging</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>DFS</td>
<td>disease free survival</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazaacyclododecanetetraacetic acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>ECD</td>
<td>extracellular domain</td>
</tr>
<tr>
<td>EGF</td>
<td>human epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>human epidermal growth factor receptor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPR</td>
<td>enhanced permeability and retention effect</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ErbB</td>
<td>erythroblastic leukemia viral oncogene</td>
</tr>
<tr>
<td>Fab</td>
<td>monovalent antigen-binding fragment of an antibody</td>
</tr>
<tr>
<td>F(ab′)2</td>
<td>divalent antigen-binding fragment of an antibody</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FeRγ</td>
<td>Fc gamma receptor</td>
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<td>neonatal Fc receptor</td>
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<td>fine needle aspiration</td>
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<tr>
<td>FoV</td>
<td>field of view</td>
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<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
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<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>GLUT</td>
<td>glucose transporter</td>
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<tr>
<td>HAMA</td>
<td>human anti-mouse antibody</td>
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<tr>
<td>HERA</td>
<td>the herceptin adjuvant trial</td>
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<td>HER1</td>
<td>human epidermal growth factor receptor 1</td>
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<td>IRF</td>
<td>immunoreactive fraction</td>
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<tr>
<td>ITLC-SG</td>
<td>instant thin layer-silica gel chromatography</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo dalton</td>
</tr>
<tr>
<td>keV</td>
<td>kilo electron volt</td>
</tr>
<tr>
<td>Ki67</td>
<td>cellular marker of proliferation, named from the city of origin (Kiel, Germany) and the number of the original clone in the 96-well plate</td>
</tr>
<tr>
<td>LABC</td>
<td>locally advanced breast cancer</td>
</tr>
<tr>
<td>LCIS</td>
<td>lobular carcinoma in situ of the breast</td>
</tr>
<tr>
<td>LI</td>
<td>localization index</td>
</tr>
<tr>
<td>LOR</td>
<td>line of response</td>
</tr>
<tr>
<td>LSO</td>
<td>lutetium oxyorthosilicate</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MBC</td>
<td>metastatic breast cancer</td>
</tr>
<tr>
<td>MBq</td>
<td>megabequerels</td>
</tr>
<tr>
<td>mCi</td>
<td>millicurie</td>
</tr>
<tr>
<td>MDR1</td>
<td>multidrug-resistance gene</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MRM</td>
<td>modified radical mastectomy</td>
</tr>
<tr>
<td>MUC</td>
<td>cell surface-associated mucin</td>
</tr>
<tr>
<td>muMAb</td>
<td>murine monoclonal antibody</td>
</tr>
<tr>
<td>N9831</td>
<td>the north central cancer treatment group trial</td>
</tr>
<tr>
<td>NaHCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>NaH&lt;sub&gt;2&lt;/sub&gt;P&lt;sub&gt;4&lt;/sub&gt;</td>
<td>sodium phosphate</td>
</tr>
<tr>
<td>NaI(Tl)</td>
<td>sodium iodide crystal doped with thallium</td>
</tr>
<tr>
<td>NaN&lt;sub&gt;3&lt;/sub&gt;</td>
<td>sodium azide</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>NPV</td>
<td>negative predictive value</td>
</tr>
<tr>
<td>NSABP B31</td>
<td>The National Surgical Adjuvant Breast and Bowel Project Trial B-31</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
</tr>
<tr>
<td>ORR</td>
<td>objective response rate</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>p27&lt;sup&gt;kip1&lt;/sup&gt;</td>
<td>cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pCR</td>
<td>pathological complete response</td>
</tr>
<tr>
<td>PD</td>
<td>progressive disease</td>
</tr>
<tr>
<td>PDK1</td>
<td>pyruvate dehydrogenase kinase 1</td>
</tr>
<tr>
<td>PEM</td>
<td>positron emission mammography</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>Pgp-170</td>
<td>p-glycoprotein 170</td>
</tr>
<tr>
<td>p.i.</td>
<td>post-injection</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIB</td>
<td>para-iodobenzoate</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PPV</td>
<td>positive predictive value</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RCP</td>
<td>radiochemical purity</td>
</tr>
<tr>
<td>RECIST</td>
<td>response evaluation criteria in solid tumours</td>
</tr>
<tr>
<td>ROI</td>
<td>region-of-interest</td>
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<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>scFv</td>
<td>single chain variable region fragment</td>
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<tr>
<td>SCCHN</td>
<td>squamous cell carcinoma of the head and neck</td>
</tr>
<tr>
<td>SD</td>
<td>stable disease</td>
</tr>
<tr>
<td>SE-HPLC</td>
<td>size-exclusion high performance liquid chromatography</td>
</tr>
<tr>
<td>SPECT</td>
<td>single photon emission computed tomography</td>
</tr>
<tr>
<td>SUR</td>
<td>standard uptake ratio</td>
</tr>
<tr>
<td>SUV</td>
<td>standard uptake value</td>
</tr>
<tr>
<td>T/B</td>
<td>tumour-to-blood ratio</td>
</tr>
<tr>
<td>T/NT</td>
<td>tumour-to-normal tissue ratio</td>
</tr>
<tr>
<td>TAG-72</td>
<td>tumour-associated glycoprotein 72</td>
</tr>
<tr>
<td>TDLU</td>
<td>terminal ductal-lobular unit</td>
</tr>
<tr>
<td>TGF-α</td>
<td>transforming growth factor alpha</td>
</tr>
<tr>
<td>TGI</td>
<td>tumour growth index</td>
</tr>
<tr>
<td>TK</td>
<td>tyrosine kinase</td>
</tr>
<tr>
<td>TNM</td>
<td>tumour, node, metastasis</td>
</tr>
<tr>
<td>t&lt;sub&gt;R&lt;/sub&gt;</td>
<td>retention time</td>
</tr>
<tr>
<td>TTP</td>
<td>time to disease progression</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEFGR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VOI</td>
<td>volume-of-interest</td>
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CHAPTER ONE

Introduction
1.1 Current status in the detection, diagnosis, and treatment of breast cancer

1.1.1 Breast cancer epidemiology and etiology

In 2009, an estimated 22,700 Canadian women and 200,000 American women will be diagnosed with breast cancer (BC), confirming this disease as the most common epithelial cancer and second only to lung cancer as the leading cause of cancer related-mortality among women in North America (1-3). The lifetime risk of developing BC for women in North America is approximately 1 in 8 and despite significant advances in the diagnosis and treatment of BC, approximately 20% of women will eventually die from their disease (1, 2, 4). Incidences of BC increase with advancing age. The probability of developing invasive BC before the age of 40 is only 0.48%, this increases to 3.86% between the ages of 40 and 59 and to 6.95% over the age of 70 (1). Over the last two decades, there has been a steady decline in BC-related mortality, which is attributed to earlier diagnosis through effective screening programs as well as access to high quality treatment.

The vast majority of BCs are sporadic and the cause of BC in individual patients is not known, however, there are many risk factors associated with an increased incidence of the disease. Risk factors include gender, age, late age of first pregnancy, family history, race, exposure to radiation, exposure to estrogen, and genetic predispositions (e.g. mutations in genes such as BRCA1, BRCA2), as well as lifestyle factors such as excessive alcohol consumption and obesity after menopause (5, 6).

1.1.2 Detection, diagnosis and staging of breast cancer

Breast cancer originates in the mammary gland, the functional structure of the adult breast. Most BCs originate from the epithelial cells of the terminal ductal-lobular unit (TDLU), comprised of the lobule and adjacent ducts (7). There currently exist two models for the development of BC. The first suggests that each tumour subtype evolves from a distinct cell of origin, while the second model suggests that the cell of origin can be the same for different subtypes and the tumour phenotype is determined by acquired genetic and epigenetic events (8). While the development of BC is not fully understood, evidence suggests that the occurrence of atypical
ductal hyperplasia, atypical lobular hyperplasia, lobular carcinoma *in situ* (LCIS) and ductal carcinoma *in situ* (DCIS) increases one’s risk of developing invasive carcinoma (6, 9). Ductal carcinoma *in situ* (DCIS) accounts for 15-20% of newly diagnosed cases of BC. Additionally, the most commonly diagnosed type of BC is invasive ductal carcinoma (also known as infiltrating ductal carcinoma), which accounts for 75-85% of invasive BCs.

Breast cancer is diagnosed through a combination of clinical examination, mammography or other diagnostic imaging (see section 1.6), and histopathological analysis of a primary lesion biopsy, collected by core needle biopsy (CNB), fine needle aspiration (FNA), or other excision methods. Following diagnosis, the prognosis and treatment of an individual patient is determined by staging and determination of pathologic features such as receptor status and tumour grade. Breast cancer is staged by the tumour, node, metastasis (TNM) system published by the American Joint Committee on Cancer (AJCC) (10). The TNM system focuses on prognostic factors associated with recurrence, metastasis and death, characterizing BC by: tumour size (T), the number and location of lymph nodes involved (N), and the presence or absence of distant metastatic disease (M) (10). Stage 0, the earliest stage (T_{is}, N_{0}, M_{0}), represents *in situ* disease and stage IV (any T, any N, M_{1}) represents metastatic breast cancer (MBC), the most advanced stage of disease (10). Between stage 0 and IV, stages I, IIA, IIB, IIIA, IIB, and IIIC classify tumours with increasingly poor prognostic risk. For patients with stage II and III BC, determination of lymph node involvement is the single most important prognostic factor, as node-positive BCs have a significantly higher likelihood of recurrence and development of distant metastases (6). Other important prognostic information comes from histopathological grading of the tumour. The most widely used histologic grading system of BC is the Nottingham combined histologic grade (Elston-Ellis modification of Scarff- Bloom- Richardson grading system) (11, 12). Tumours are graded for the percentage of tubule formation, the degree of nuclear pleomorphism and their mitotic index (13). Each feature is graded out of 3 and the scores are summed to give a total out of 9, where 3-5 is low grade, 6-7 is intermediate and 8-9 is high grade. Despite not being including in the AJCC staging system, both the World Health Organization and the College of American Pathologists have recognized histological grading as an important prognostic factor for BC (11, 12).

### 1.1.3 Treatment of breast cancer

Management of BC is transdisciplinary involving clinical examination, imaging diagnostics (see section 1.6), pathology, surgery, radiation oncology, and medical oncology. For an individual
patient, the choice of treatment depends on the stage of the disease, the grade and biological characteristics, as well as the patient’s age, health, and personal preference. Most treatment regimes begin with some form of surgery for local disease control, followed by local and/or systemic adjuvant therapies. Different types of surgery include: breast conserving surgery (BCS) and modified radical mastectomy (MRM) (6).

DCIS (Stage 0), the earliest stage of disease, usually has a good prognosis and treatment commonly involves BCS, followed by local radiation therapy, except in cases of low grade disease (6). Additionally, tamoxifen, a selective estrogen receptor modulator, has been shown to reduce progression to invasive BC and recurrence of non-invasive disease by 43% and 31%, respectively, following BCS and radiation in patients with estrogen receptor (ER)-positive DCIS (14).

For early stages (stages I and II) of invasive BC, treatment is often BCS and axillary node dissection, followed by radiation therapy with or without tamoxifen, depending on estrogen-receptor status (15). In some cases, such as large tumour size, mastectomies may be recommended, however, preoperative chemotherapy (neoadjuvant chemotherapy) can be utilized to shrink large primary tumours and allow for BCS (16). Adjuvant chemotherapy consisting of cyclophosphamide, methotrexate and 5-fluorouracil (CMF) or doxorubicin and cyclophosphamide (AC) or cyclophosphamide, epirubicin and 5-fluorouracil (CEF) is recommended for patients with positive lymph node involvement (17). Adjuvant chemotherapy consisting of CMF or AC is also recommended for high-risk node-negative patients (18).

Since the advent of mammographic screening, the incidence of locally advanced breast cancer (LABC; stage III) at diagnosis has diminished, but remains a significant clinical challenge. LABC is associated with a worse prognosis and long-term survival of approximately 50% (19). The management of LABC requires combined modality treatment often involving surgery, radiotherapy and systemic therapy. Stage III disease is divided into large operable tumours (IIIA) and inoperable tumours (IIIB and IIIC). There are generally two approaches to treating stage IIIA BC: the first is MRM followed by adjuvant systemic therapy and locoregional radiotherapy and the second is preoperative chemotherapy followed by surgery and radiotherapy (19). Occasionally preoperative chemotherapy results in tumour shrinkage, allowing for BCS, however results from two trials suggest that patients whose tumours were
down-staged to allow BCS when it was not initially planned were at higher risk for local recurrence and reduced survival, therefore this strategy is not recommended for LABC (20, 21). There are numerous chemotherapy regimens, however, current Canadian guidelines recommend that chemotherapy for operable stage III BC should contain an anthracycline (19). Taxanes are currently under investigation for operable stage III and stage II, however these agents are not currently included in the Canadian guidelines (19).

It is generally recommended that patients with inoperable stage IIIB or IIIC, including inflammatory BC be treated with anthracycline-based chemotherapy (19). A CMF regime is recommended for patients who cannot tolerate anthracyclines. Multivariate analysis of several studies has shown that a complete response following primary chemotherapy correlates with improved disease free survival (DFS) and overall survival (OS) (19). Unresponsive tumours may be treated with second-line chemotherapy using taxanes. Following chemotherapy, responsive patients proceed to locoregional therapy including irradiation and MRM, if possible. Two studies have demonstrated a benefit to MRM and radiation therapy compared to either treatment alone (22, 23).

Additionally, hormone therapy with tamoxifen improves survival of ER-positive patients and is recommended for 5 years for all stage III ER-positive tumours, including those who are not candidates for chemotherapy (19). The aromatase inhibitor, anastrozole, has been compared with tamoxifen in patients with early BC and early results indicate improved DFS and fewer side effects (24).

Patients with BC metastasis to distant sites, such as the bones, lungs, liver, or brain, are classified as stage IV or having advanced BC (25). Advanced BC is associated with a median survival of 2-3 years and prognosis is dependant on the site of organ metastasis, with visceral involvement carrying a worse prognosis (3). Treatment of the majority of patients with advanced BC is palliative in nature (i.e. cure is not expected, but symptoms of the disease can be alleviated with treatment). Systemic combination chemotherapy is the primary treatment (25). Endocrine therapy is also beneficial for hormone responsive tumours.

In addition to traditional systemic therapies, biological therapy has also been shown to improve DFS and OS in subsets of patients. HER2-overexpression occurs in approximately 20% of BCs and is associated with a poor clinical outcome (26). Trastuzumab (Herceptin®, Genentech, Inc., CA), a monoclonal antibody therapy directed against HER2 has shown clinical efficacy in early and advanced stages of BC by prolonging DFS and OS in HER2-positive patients when administered alone or in combination with chemotherapy (see section 1.2) (27).
1.2 Trastuzumab in the management of HER2-overexpressing breast cancer

1.2.1 HER2 biology and clinical significance

The human epidermal growth factor receptor 2 (HER2) also known as HER-2/neu or ErbB2, is a member of the ErbB family of cell surface receptor tyrosine kinases (RTKs) involved in the transmission of signals controlling normal cell growth and differentiation (28). The family consists of 4 receptors sharing a high degree of homology; HER1 (EGFR, ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). The HER2 receptor, a 185 kDa type 1 transmembrane glycoprotein, is encoded by the HER2 gene, a proto-oncogene located on the long arm of chromosome 17q21 (29). The erbB receptors share a similar structure comprised of a cysteine-rich extracellular region, a lipophilic transmembrane segment, an intracellular domain with tyrosine kinase activity and a carboxy terminal domain that is autophosphorylated upon receptor activation (30). The receptors exist as monomers which upon ligand binding associate to form of homo- or heterodimers, resulting in the activation of intrinsic kinase activity, ultimately leading to stimulation of intracellular signaling cascades (31). Notably, HER2 is the preferred dimerization partner of the other HER-family receptors (32) and HER2-containing heterodimers result in potent mitogenic signaling, especially HER2:HER3 heterodimers (31, 33, 34). The preference for HER2-containing dimers is likely due to the open conformational state of HER2 compared with the other receptors. Elegant crystallographic studies have revealed that HER1, HER3 and HER4 exist in a closed conformation with the dimerization domain (domain II) tethered to domain IV preventing the formation of dimers with other HER receptors (35-37). Upon ligand binding to domains I and III the receptors undergo a significant conformational rearrangement exposing the previously concealed dimerization arm for interaction with other HER receptors also in the active state (35, 37, 38). In contrast, HER2 is an “orphan receptor” with no known endogenous ligands, however, this receptor is constitutively in a open conformation with the dimerization arm exposed, resembling the ligand-bound state of EGFR (35, 38). This open-confirmation facilitates dimerization with other HER family receptors defining a key role for HER2 in the signal transduction of ligand-driven heterodimeric complexes.

HER2 is normally expressed in low levels in adult cell types including the breast epithelium, the central nervous system, bone, muscle, skin, heart, lungs and intestinal epithelium.
The function of HER2 in adult tissues is not fully understood, however the receptor appears to play a role in the proliferation and differentiation of epithelial cells (31) and in the protection of cardiomyocytes against apoptosis (40). In fetal tissues, however, HER2 is widely expressed and is critical to normal development (39).

The clinical significance of HER2 in cancer was first discovered in the early 1980s following the identification of the *neu* oncogene, the mutationally active rat homologue of HER2 (41). The human homologue was soon identified (29, 42) and found to be overexpressed in a mammary carcinoma cell line (43). On the basis of these findings, Slamon and co-workers retrospectively examined HER2 expression in a series of primary human breast tumours and reported a significant association between HER2 overexpression, relapse and patient survival (26). HER2 is now known to be overexpressed in approximately 20% of BCs (44), and overexpression is also common in ovarian, prostate, lung, gastric and oral cancers (45).

Overexpression of HER2 in BC is primarily due to gene amplification resulting in increased transcription and protein expression, but may also result from post-transcriptional dysregulation such as increased protein translation (46). Preclinical studies suggest that the association between HER2 and malignant growth is not incidental. Breast cancer cells may express HER2 at levels 10- to 100-fold greater than normal epithelial tissues (47). This high density of HER2 promotes the formation of HER2 heterodimers as well as the formation of ligand-independent constitutively active HER2 homodimers (33, 46, 48). HER2-containing dimers persist longer on the cell surface due to their slower rate of internalization, resulting in overactive cell signaling and leading to dysregulated cell growth, proliferation and malignant transformation (33). Pathologically, HER2 overexpression is associated with a high S-phase fraction, absence of ER/PR expression, presence of nodal metastasis, high nuclear grade and ductal (as opposed to lobular) histology (49). Clinically, HER2 is associated with aggressive disease, increased risk of relapse and poor long-term survival (26, 49).

Apart from its prognostic significance, HER2 is also a predictive factor in BC. HER2 is associated with partial resistance to hormone therapy, as well as improved response or resistance to certain chemotherapeutic agents (44, 50, 51). Furthermore, HER2 overexpression is a significant predictive factor of response to the HER2-targeted monoclonal antibody trastuzumab (Herceptin®) (44).
1.2.2 Monoclonal antibody trastuzumab (Herceptin®)

The HER2 receptor is a desirable therapeutic target for several reasons (see section 1.2.1). Given the critical role of HER2 in the oncogenesis and progression of BC, extensive research has focused on developing HER2-targeted cancer therapeutics. Trastuzumab, a recombinant humanized monoclonal antibody (mAb) is the first HER2-targeted therapy approved by the U.S. Food and Drug Administration (FDA) and Health Canada for treatment of HER2-overexpressing BC. Trastuzumab binds to the C-terminal portion of the extracellular domain (ECD) of HER2 near the juxtamembrane region in domain IV (36). Important preclinical in vitro and in vivo studies of the murine monoclonal antibody (muMAb) precursor, 4D5, showed the potential therapeutic benefit of the antibody for treatment of BC (52-55), however, in a preclinical study in monkeys, a monkey anti-mouse antibody (MAMA) response significantly altered the pharmacokinetics of muMAb4D5, thereby limiting the therapeutic efficacy of the drug (56). Additionally, in a phase I trial of muMAb4D5 a human anti-mouse antibody (HAMA) response was encountered (57). Therefore, a humanized version of muMAb4D5, known as trastuzumab, was subsequently engineered by grafting the muMAb4D5 antigen binding loops of the hypervariable complementary determining regions (CDRs) to a human IgG1 framework (58). In vitro and in vivo studies of humanized MAb4D5 (trastuzumab) in HER2-positive BC cell lines and xenograft models in immunodeficient mice demonstrated anti-tumour activity as a monotherapy and enhanced activity in combination with various chemotherapeutic agents including, paclitaxel, doxorubicin, cisplatin thiotepa, etoposide, methotrexate and vinblastine (58-61).

1.2.3 Summary of the clinical trials with trastuzumab

In the early 1990’s, only 10 years after the discovery of the HER2 oncogene, the toxicity, pharmacokinetics, and response rate to trastuzumab were evaluated in single-agent phase II clinical trials in patients with advanced HER2-positive MBC previously treated with chemotherapy (62, 63). Of these studies, the larger, pivotal Phase II trial evaluated 222 extensively pre-treated patients with HER2-positive MBC treated with 4 mg/kg trastuzumab, followed by 2 mg/kg weekly until disease progression (63). The objective response rate (ORR) was 15% and the median duration of response was 9.1 months. Furthermore, target serum
concentrations (≥20 µg/mL) were maintained in greater than 90% of patients and no patients developed an immune response (63). The most clinically significant adverse event was cardiac dysfunction, which occurred in 4.7%. When follow-up data were included, this rate rose to 8.5%. Interestingly, >90% of these patients had received prior anthracycline therapy. It is thought that trastuzumab may exacerbate the known cardiotoxicity associated with this class of chemotherapeutics (64), however trastuzumab-mediated cardiotoxicity appears to be at least partially reversible upon stopping therapy, often without the need for medical intervention (65). This trial led to the approval of trastuzumab monotherapy for HER2-positive MBC in patients previously treated with chemotherapy (27).

Single-agent trastuzumab was later evaluated as first-line treatment for patients with HER2-positive MBC. In these studies patients were treated with either 4 mg/kg followed by weekly doses of 2 mg/kg or 8 mg/kg followed by 4 mg/kg bi-weekly (66) or 8 mg/kg followed by 6 mg/kg at 3-week intervals (67) and ORRs were 26% and 19%, respectively.

A significant improvement in response was observed when trastuzumab was administered in combination with chemotherapy. A randomized Phase III trial in 469 patients with MBC compared first-line treatment of trastuzumab in combination with chemotherapy (n=235) vs. chemotherapy alone (n=234) (68). Chemotherapy consisted of anthracycline (doxorubicin 60 mg/m² or epirubicin 75 mg/m² plus cyclophosphamide 600 mg/m² [AC]) or paclitaxel 175 mg/m² if the patient had received prior adjuvant AC treatment. Primary endpoints were time to disease progression (TTP) and safety. The addition of trastuzumab to AC or paclitaxel was associated with a significantly longer TTP than chemotherapy alone (median, 7.4 vs. 4.6 months; P<0.001), higher ORR (50% vs. 32%; P<0.001), longer duration of response (median, 9.1 vs. 6.1 months; P<0.001) and longer survival (median, 25.1 vs. 20.3 months; P=0.01) (68). On the basis of these clinical trials, trastuzumab received FDA approval for use in patients with HER2-positive MBC both as first-line therapy in combination with paclitaxel and as a single agent for patients who have received one or more chemotherapy regimen. Cardiotoxicity was more prevalent with the combined treatment, especially in patients treated previously or concurrently with AC. These results led to the recommendation that concomitant anthracyclines and trastuzumab be avoided, and provided a rationale to evaluate the safety and efficacy of other non-anthracycline containing trastuzumab-based regimens. A more recent Phase III clinical trial confirmed the benefit of trastuzumab in combination with non-anthracycline containing chemotherapy in first-line treatment of 186 HER2-positive MBC patients (69). In this study, cardiotoxicity was minimal, with only 1 patient in the combination...
arm experiencing symptomatic heart failure. Numerous smaller Phase II trials have similarly evaluated trastuzumab in combination with non-anthracycline chemotherapy for first, second or third-line treatment of HER2-positive MBC patients (27).

Following the success of trastuzumab in the treatment of MBC, four large randomized clinical trials have evaluated the use of trastuzumab in the adjuvant setting of early-stage HER2-positive BC. The National Surgical Adjuvant Breast and Bowel Project trial B-31 (NSABP B31) and The North Central Cancer Treatment Group trial (N9831) compared doxorubicin and cyclophosphamide followed by paclitaxel alone or in combination with trastuzumab for 52 weeks following surgery of node positive or high-risk node-negative operable BC (70). An interim analysis was performed after 3 years with a median follow up of 2 years. The trastuzumab-containing regime was associated with 12% greater DFS and a 33% reduction in mortality (70). Interim analysis of 2 other ongoing studies, the HERceptin Adjuvant (HERA) trial and The Breast Cancer International Research Group trial (BCIRG 006) have demonstrated higher DFS and OS in trastuzumab-containing regimens compared to chemotherapy alone (71-73). Notably, adverse cardiac events were higher in anthracycline-containing regimens, with the highest incidence occurring in regimens combining anthracycline and trastuzumab, however there were no cardiac-related mortalities (74). These trials led to the FDA and Health Canada approval of trastuzumab for the use of adjuvant therapy in early-stage BC.

Trastuzumab has also been evaluated in the neo-adjuvant setting. Budzar and co-workers, describe a randomized phase III trial evaluating paclitaxel followed by 5-fluorouracil (5-FU) alone or in combination with trastuzumab in patients with HER2-positive operable BC (stages II-IIIA) (75). Following the early observation of a high pathological complete remission (pCR) rate in the first 42 patients, the study protocol was revised to discontinue the chemotherapy alone regimen and recruit an additional 21 patients to the trastuzumab-containing treatment arm. At a median follow-up time of 3 years, pCR and DFS were significantly higher for the trastuzumab treatment group (75). In another phase II trial evaluating preoperative trastuzumab in combination with docetaxel and carboplatin compared to chemotherapy alone in HER2-positive advanced BC, preliminary results indicate that pCR was higher for the trastuzumab-containing regimen (36.6% vs. 9%) (76). While no adverse cardiac events have
been reported in these treatment regimens, long-term safety and efficacy remain undefined for trastuzumab in the neoadjuvant setting (75, 76).

1.2.4 Current status of trastuzumab in the treatment of breast cancer

1.2.4.1 Approved indications of trastuzumab in breast cancer treatment

Based on extensive clinical testing, trastuzumab is currently approved by the FDA and Health Canada for first-line treatment of HER2-positive MBC in combination with paclitaxel, as well as monotherapy for second or third-line treatment of HER2-positive MBC. Trastuzumab has also been approved by the FDA in combination with doxorubicin, cyclophosphamide and paclitaxel for the adjuvant treatment of patients with early-stage HER2-positive, node-positive BC, as well as a single-agent following adjuvant multi-modality anthracycline-based therapy in patients with early-stage HER2-positive, node-positive or high-risk node-negative BC (patients with one or more high-risk features such as hormone receptor–negative tumours, grade 2/3, tumours larger than 2 cm, or in patients younger than 35 years). In 2008, the FDA approved two new trastuzumab-containing regimens for the adjuvant treatment of early-stage HER2-positive, node-positive or high-risk node-negative BC. These include (1) docetaxel, carboplatin and trastuzumab (TCH) and (2) doxorubicin, cyclophosphamide, trastuzumab and docetaxel (AC-TH) (77).

1.2.4.2 Recommended guidelines for selection of patients for trastuzumab therapy

Guidelines set by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) recommend that all patients with invasive BC be evaluated for HER2-positivity for eligibility for trastuzumab-based therapy regimes. HER2-positivity is defined on the basis of HER2 protein overexpression or gene amplification (44). HER2 protein overexpression is measured by immunohistochemical analysis (IHC) of excised tumour tissue and graded using a 4-point scale of increasing receptor expression, where 0 and 1+ are HER2-negative, 2+ is considered equivocal and 3+ is positive. A score of 3+ is defined as complete, uniform, intense membrane staining of >30% invasive tumour cells and 2+ is defined by complete membrane staining that is either nonuniform or weak in intensity but with obvious circumferential distribution in at least 10% of tumour cells (44, 78). Gene amplification is most commonly measured by fluorescence in situ hybridization (FISH) and most often scored as the ratio of HER2 to chromosome centromere 17 (HER2:CEP17) (44, 78). A ratio of >2.2 is considered positive for gene amplification, while a ratio of 1.8 to 2.2 is considered equivocal
and $<1.8$ is negative. Patients are considered HER2-positive and eligible for trastuzumab-based therapy with IHC scores of 3+ or 2+ and FISH positivity.

Recent ASCO/CAP guidelines for HER2 testing emphasizes the importance of standardized and validated procedures to minimize variability and sampling errors (44). The HercepTEST (DAKO, Glostrup, Denmark) and Pathway (Ventana, Tucson, Arizona) are two FDA-approved IHC assays for the detection of HER2-overexpression (44). Similarly, the PathVysion HER2 DNA probe (Vysis, Downers Grove, Illinois) and INFORM HER2/neu probe (Ventana) are two FDA-approved assays for the detection of HER2 gene amplification.

The current guidelines vary from those used during many of the early trastuzumab clinical trials (44, 66, 68). As such, some of these trials included patients with tumours graded as 2+, FISH-negative. Furthermore, the definition of 3+ was previously defined as moderate to strong complete membrane staining in $>10\%$ of tumour cells. The updated selection criterion suggests that patients included in those early clinical trials may now be considered HER2-negative. Although these studies still demonstrated significant benefit in the selected patient population, retrospective analysis has shown that the beneficial effects of trastuzumab appear to be greater in FISH-positive patients (79). Notably, the majority of FISH-negative samples were from patients scored as 2+ by IHC. Furthermore, in a study by Vogel et al there was an ORR of 35% for patients whose tumours were scored as 3+ while those scored as 2+ were unresponsive (66). The new guidelines are expected to reduce uncertainty in equivocal cases.

While HER2 overexpression is a valuable predictive factor for response to trastuzumab-based therapy, it remains insufficient for predicting response in individual patients. Further understanding of the mechanisms of action and resistance to trastuzumab is necessary to optimize patient selection for this drug.

1.3 The mechanisms of action of trastuzumab

The mechanisms of action of trastuzumab are not fully understood. Significant \textit{in vitro} and \textit{in vivo} data describing multiple mechanisms of action suggest that the mode of action is multifactorial and complex (80). It is currently not possible to determine which mechanisms are contributing to therapeutic benefit in individual patients.
1.3.1 Promotion of an immune-mediated response

Trastuzumab was intentionally engineered as a humanized IgG1 as it is the preferred isotype for supporting antibody-dependent cellular cytotoxicity (ADCC) (58). ADCC is primarily mediated by the activation of natural killer cells (NK) bearing the Fc gamma receptor (FcRγ), which recognizes the Fc domain of antibodies such as trastuzumab, leading to lysis of trastuzumab-bound cancer cells (80). Clynes et al. demonstrated the importance of ADCC for the in vivo efficacy of trastuzumab in a tumour response study with FcRγ wildtype (FcRγ+/+) and knockout mice (FcRγ-/-) (81). In this study, trastuzumab treatment resulted in a tumour regression rate of 96% in the wild type mice bearing BT-474 HER2-overexpressing tumours compared to only 29% tumour regression in the knockout mice (81). Some limited evidence of ADCC as a possible mechanism of action of trastuzumab has also been demonstrated in patients receiving the drug preoperatively in which those with complete or partial remission were found to have higher in vivo tumour infiltration of leucocytes with a higher capability of mediating in vitro ADCC (82). Recently, Beano et al found an association between progression-free survival and NK activity in HER2-positive, MBC patients treated with second-line trastuzumab monotherapy, supporting reports of an immunological effect of trastuzumab (83).

1.3.2 Inhibition of angiogenesis

Another mechanism that appears to be employed by trastuzumab is the inhibition of angiogenesis. Angiogenesis, the process of new blood vessel formation, is essential for continued tumour growth beyond a few millimeters as well as metastasis. Angiogenesis is stimulated by a variety of growth factors and regulatory molecules, including vascular endothelial growth factor (VEGF). Overexpression of HER2 in human tumour cells is closely associated with increased expression of VEGF (84). In mice bearing human HER2-overexpressing BC tumours, treatment with trastuzumab resulted in normalization and regression of the vasculature (85), as well as reduced expression of VEGF, transforming growth factor-α (TGF-α), angiopoietin-1 (Ang-1) and plasminogen activator inhibitor-1 (PAI-1), which normally act to promote angiogenesis (85, 86). Additionally, blood vessels in the trastuzumab-treated tumours more closely resemble those of a normal phenotype (85). Izumi and co-workers suggest that as the tumour vascular network becomes more organized and efficient following trastuzumab therapy, this may allow improved delivery of drugs to previously inaccessible regions (85). Indeed, a study in mice bearing HER2-overexpressing BC xenografts found a greater reduction in microvessel density and greater anti-tumour effect in mice treated with
paclitaxel in combination with trastuzumab compared to paclitaxel alone (86). Recently a clinical study found an association between microvessel density, measured by light microscopy of a core needle biopsy, and HER2 expression in tissue from primary BC, but the effects of trastuzumab on angiogenesis in patients remains to be proven (87).

1.3.3 Inhibition of HER2-ECD cleavage

Several *in vitro* and *in vivo* studies have shown that the HER2 receptor undergoes proteolytic cleavage, resulting in the release of a soluble 110 kDa ECD and a 95 kDa constitutively active amino-terminally truncated membrane-bound fragment (p95) (88-93). Circulating levels of HER2-ECD can be detected in the serum of BC patients using an FDA-approved enzyme-linked immunoassay kit (ELISA) and have prognostic significance in patients with early and MBC (94-96). Elevated levels of circulating HER2-ECD are associated with high histological grade, nodal involvement, ER/PR-negativity, worse disease-free survival and reduced survival (94-96). Trastuzumab has been shown to block metalloprotease-mediated cleavage of HER2-ECD in SKBr-3 and BT-474 HER2-overexpressing BC cells (92). Additionally, clinical studies have demonstrated an association between declining levels of HER2-ECD, measured using FDA-approved automated ELISA assays (Bayer Immuno1 or ADVIA Centaur™), with improved response rates and progression-free-survival following trastuzumab therapy, indirectly supporting the hypothesis that trastuzumab may act by inhibiting HER2 cleavage (97, 98).

1.3.4 Inhibition of PI3K pathway

HER2 overexpression leads to the formation of excessive HER2 homo- and heterodimers, which activate multiple cell signaling pathways, including the phosphoinositide 3-kinase (PI3K) pathway, partially responsible for cell growth and survival (31, 99). Receptor dimerization results in phosphorylation of the tyrosine kinase domain, which serves as a docking site for adaptor proteins and initiates signaling cascades (99). Following receptor activation, PI3K is recruited to the cell membrane were it phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) activating messenger proteins such as pyruvate dehydrogenase kinase 1 (PDK1) and Akt. Once activated, Akt mediates the activation and inhibition of several targets, resulting in cellular survival, growth and proliferation (99). The lipid phosphatase and tensin homolog (PTEN) is a PIP3 phosphatase,
which negatively regulates the PI3K/Akt pathway (99). The non-receptor tyrosine kinase Src is also activated by phosphorylated tyrosines and inhibits the phosphatase activity and growth-inhibitory effects of PTEN. Nagata et al. have demonstrated that interaction between HER2 and Src is disrupted in response to trastuzumab treatment resulting in Src inactivation and subsequent activation of PTEN (100). Increased activity of PTEN results in rapid dephosphorylation of Akt, inhibiting cell proliferation (99, 100).

### 1.3.5 Promotion of HER2-receptor downregulation

Diminished receptor signaling may also result from trastuzumab-mediated internalization and degradation of the HER2 receptor, leading to a reduction in surface receptor density. Whether or not trastuzumab results in receptor downregulation is currently a subject of controversy. Several *in vitro* studies have demonstrated that treatment of HER2-overexpressing breast and ovarian cancer cell lines with trastuzumab results in downregulation of HER2 (92, 101-109). It has been proposed that trastuzumab-mediated receptor downregulation is the result of accelerated endocytic degradation (107, 109), however other studies have shown that HER2 is excluded from clatherin-coated pits and does not undergo endocytosis (110, 111). Furthermore, the latter studies suggest that HER2 is an internalization-resistant receptor and trastuzumab does not induce receptor downregulation (110, 111). It should be noted, however, that these internalization studies were performed on HER2-overexpressing cells following only 1 hour incubation with trastuzumab and it is possible that this time point is not sufficient to observe detectable levels of internalized receptors. Mittendorf et al have shown that trastuzumab-mediated receptor internalization is detectable by flow cytometry only after 12 hours of incubation with the antibody (106). Additionally, Austin et al have reported that HER2 is a dynamic, continually recycling receptor, similar to EGFR, but with a longer half-life of 19 h, therefore it is unlikely that significant levels of HER2 would be internalized after 1 h (112).

In clinical studies, only small subgroups of patients have demonstrated significant changes in HER2 expression following neoadjuvant treatment with trastuzumab (82, 105, 113, 114). The majority of patients retain their HER2 status following treatment. It is possible that the neoplastic tissue analyzed after treatment could be composed of cells that do not respond to treatment. Additionally, it is possible that IHC is insufficiently sensitive to detect these changes in HER2 density. Despite conflicting data, there are numerous studies which support downregulation of HER2 as a possible mechanism of action of trastuzumab, however the
process by which trastuzumab downregulates cell surface HER2 and its relationship with response requires further study.

1.3.6 Induction of cell cycle arrest through post-translational upregulation of $p27^{kip1}$

The cyclin-dependent kinase inhibitor $p27^{kip1}$ plays an important role in controlling cell cycle progression. Low expression of $p27^{kip1}$ is associated with excessive cell proliferation and is common in many types of cancer, including BC (115). Furthermore, low expression of $p27^{kip1}$ correlates inversely with HER2 expression (116). In vitro studies have shown that trastuzumab treatment of HER2-overexpressing BC cells increases the half-life of $p27^{kip1}$ by reducing expression of proteins involved in the sequestration of $p27^{kip1}$, such as cyclin D1, and by blocking ubiquitin-dependent degradation of $p27^{kip1}$ (117). Association of $p27^{kip1}$ with cyclin-dependent kinase-2 (cdk2) results in cdk2 inactivation and G1 arrest, resulting in an increased percentage of cells in G0/G1 phase and a decrease of cells in S phase (117-119). No cell cycle effects were detected using a non-inhibitory HER2 monoclonal antibody (FRP5) or low-HER2 expressing BC cells (118, 119).

1.4 De Novo and Acquired resistance to trastuzumab

Clinical trials have established that trastuzumab is active against HER-overexpressing BC, however, only a minority (12-35%) of patients respond to trastuzumab when administered as a single agent and patients who show an initial response to trastuzumab often acquire resistance to the drug in less than a year (120, 121). Substantial in vitro and in vivo studies aimed at elucidating the mechanisms of trastuzumab resistance have implicated a variety of independent pathways. Proposed mechanisms include the loss of PTEN, activation of alternative signaling pathways such as insulin-like growth factor receptor 1 (IGF-1R), expression of elevated levels of HER-family ligands such as TGF-α, masking of the trastuzumab-binding epitope by membrane proteins such as MUC4, and modulation of $p27^{kip1}$ through elevated levels of cdk2. Further elucidation and understanding of the mechanisms by which tumours escape trastuzumab-mediated toxicity will be critical to improving the management of patients with HER2-positive BC.
1.5 Pertuzumab (Omnitarg®) in the management of breast cancer

Following the success of trastuzumab, Genentech Inc. has developed a second generation HER2 antibody, pertuzumab (Omnitarg®), the first in a new class of targeted therapeutics known as dimerization inhibitors (122). Pertuzumab (recombinant mAb 2C4) is a humanized monoclonal antibody IgG1 which binds to an epitope of HER2 that is distinct from that recognized by trastuzumab, resulting in different biologic properties. Pertuzumab binds to the dimerization arm on domain II, sterically inhibiting the formation of HER2 homodimers and heterodimers with other HER family receptors (123-125). In contrast, trastuzumab binds to domain IV and does not inhibit heterodimer formation (36). As a result of this unique mechanism of action, pertuzumab has shown efficacy against HER2-overexpressing tumours, as well as low HER2-expressing lesions, where it serves as an indirect suppressor of HER3, a potent mitogenic signaling partner of HER2 (126). In preclinical studies, pertuzumab inhibits ligand-dependent growth of BC cell lines (124, 127, 128). Furthermore, the growth-inhibitory properties of pertuzumab are independent of the level of HER2 expression (124). The novel mechanism of action of pertuzumab requires the presence of HER2 but does not depend on overexpression of the receptor, suggesting that pertuzumab may be applicable to a broad range of tumour types of epithelial-cell origin, which often express HER2. Indeed, in vivo preclinical studies have demonstrated anti-tumour activity in various tumour types including BC (124, 129), colorectal carcinoma (CRC) (130), prostate cancer (124, 131), and non-small cell lung cancer (NSCLC) (129).

Clinically, pertuzumab has demonstrated therapeutic efficacy in subgroups of patients in phase II clinical trials for BC (132), ovarian cancer (133), prostate cancer (134) and NSCLC (135), and is currently being evaluated in combination with trastuzumab and docetaxel in a phase III study for metastatic HER2-positive BC (136). The reason for the selective efficacy in subgroups of patients is currently not known. Development of a test to more accurately select patients who are most likely to respond would be valuable for optimizing patient benefit. Preclinical evidence suggests that the presence of HER2:HER3 heterodimers is a better predictor of response to pertuzumab than the expression level or phosphorylation level of HER1, HER2 and HER3 (137). Further understanding of the mechanisms of action and resistance of pertuzumab is necessary to optimize patient selection for this drug.
1.6 Diagnostic imaging modalities for breast cancer

Imaging techniques are utilized for screening, detection, diagnosis, and management of BC. Current imaging modalities include X-ray mammography, ultrasonography, magnetic resonance imaging (MRI), and nuclear medicine. Each technique has advantages and disadvantages. A comprehensive discussion of the various diagnostic imaging modalities is beyond the scope of this thesis but a brief summary of the clinical utility of each modality is provided in the following sections.

1.6.1 Mammography

Mammography is the primary imaging modality for screening and diagnosis of BC. Mammography uses low-dose X-rays to examine the internal structures of the breast in order to reveal abnormalities, such as cancer. Mammography is used as a screening tool in the absence of abnormal signs or symptoms with the goal of detecting BC early, before clinical symptoms become apparent. Mammography is also used to detect and diagnose breast disease in women experiencing symptoms such as a lump or breast pain. In Canada, mammographic screening is recommended annually for women over the age of 50 (138). These recommendations are based on analysis of large, prospective clinical trials which show that screening in women aged 50-69 can reduce mortality from BC by almost a third (138). Furthermore, a retrospective analysis of 992 women with invasive cancer found that cancers detected with screening mammography were smaller and less likely to have metastasized to the lymph nodes compared to those found by clinical examination (139). Masses and microcalcifications are the most common abnormalities encountered on mammograms and their radiographic appearance provides important clues to their etiology (140). The sensitivity of mammography for detecting malignant lesions is 80-90%, but its sensitivity for detecting abnormalities in dense fibroglandular breasts is suboptimal (42-68%); however recent advances in technology have improved the sensitivity of detection in dense breasts (140, 141). A study by the National Cancer Institute has shown that digital mammography was significantly better for detection of BC in dense breasts compared to film mammography (142). Another limitation of mammography is the false-negative rate, which has been reported to be between 10-30%, indicating that a significant proportion of BCs will be occult in mammographic imaging (143).
Furthermore, mammography has low-specificity, high false-positive and low positive predictive value. Mammography can also be unreliable following surgery or radiation therapy due to limitations in distinguishing between recurrent disease and scar tissue. Mammography is a powerful screening and detection tool, but cannot rule out the possibility of BC, and often requires additional diagnostic procedures to confirm diagnosis. Currently, mammography is the only screening imaging modality associated with a decline in BC mortality.

1.6.2 Ultrasonography

Ultrasonography, also referred to as ultrasound or sonography has become an integral component to diagnostic breast imaging. Ultrasound generates real time images of internal structures using high frequency reflected sound waves. Breast ultrasound is routinely used to distinguish between cysts and solid masses with high specificity (140). Cysts, which account for 25% of breast lesions, are characterized by a round or oval shape, circumscribed margins, clearly defined posterior walls, absence of internal echoes and enhancement of distal echoes (140). In contrast, malignant masses have ill-defined margins, a height equal or greater than width and low-level heterogeneous internal echoes (140). By discriminating between features such as shape, margins and echogenicity, ultrasound in combination with mammography, is also useful in differentiating between benign and malignant breast masses. Kolb et al. demonstrated improved detection sensitivity (97% vs. 74%) of ultrasound combined with mammography compared to mammography and physical examination (144). This study also noted improved sensitivity in women with dense breasts using ultrasound compared to mammography (144). Similarly, diagnostic accuracy improved from 78% to 91% when ultrasound was performed with mammography in a trial of 2637 women with heterogeneously dense breasts, however the positive predictive value decreased from 22.6% to 11.2% (145). Advances in transducer technology have also lead to the frequent use of ultrasound in the guidance of breast biopsies (145). Other advancements, such as 3D Doppler Ultrasound, which is especially useful at characterizing blood flow and pressure in lesions, can aid in the differentiation between benign and malignant lesions, which often exhibit increased blood flow.

Limitations of ultrasound include the increased false positive rates when used as an adjunct to mammography, its inability to distinguish between some benign and malignant masses, its poor ability to visualize deep lesions, a lower sensitivity than mammography for detection of microcalcifications and the dependence of the effectiveness of the modality on the experience of the radiologist (140, 144). Currently ultrasound is most useful to guide biopsy
procedures, as well as for characterization of suspicious abnormalities as an adjunct to mammography, and for diagnosis of mammographically occult lesions, such as those in dense breasts.

1.6.3 Magnetic resonance imaging

Magnetic resonance imaging (MRI) visualizes internal structures in the body with high soft tissue resolution by measuring the emission of radio waves emitted by tissues of the body caused by the relaxation of protons in biological molecules following exposure to a strong magnetic field in combination with a radiofrequency pulse. The use of MRI for the detection of BC has advanced significantly following the introduction of dynamic contrast-enhanced MRI (DCE-MRI) which uses gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA) as a contrast agent to evaluate differences in perfusion kinetics between normal tissue, benign breast lesions and malignant tissue (143). This technique is based on the principle that malignant lesions have increased perfusion and vascular permeability, reflected by their unique uptake and washout pattern of Gd-DTPA (143). Furthermore, anatomical features such as a speculated margin, an irregular shape, rim enhancement in the presence of central enhancement, and segmental or clumped ductal enhancement are indicative of malignancy (10). Therefore, the presence and kinetics of enhancement, and the morphology of the lesion can be used to distinguish benign and malignant lesions, with positive predictive values (PPV) ranging from 76-92% (143, 146). The sensitivity of MRI for detecting invasive BC is high (88-100%), although most studies show poor specificity (143). Large scale trials of screening with MRI in unselected populations have not yet been performed, however, five prospective, non-randomized clinical trials comparing MRI and mammography in screening high risk women, defined as women with a lifetime risk of developing BC of greater than 15% such as women with familial history or BRCA1/2 mutations, have shown that the sensitivity of MRI for the detection of cancer ranged from 77% to 100%, significantly higher than that observed for mammography (25-40%) (146). The specificity of MRI in these trials was on average 5% lower than mammography, likely due to the higher false-positive rate of MRI. The results of these trials led the American Cancer Society to recommend annual MRI screening for women with a lifetime risk of developing BC of 20% and known mutation carriers (147). MRI has also been useful in planning surgical therapy in women with known BC. MRI can be used to determine
the size of tumours, extent of disease, multicentricity, and multifocality, which could greatly affect the surgical approach. Studies in women with localized BC have demonstrated that preoperative MRI identifies additional tumour foci in 11% to 31% of cases and that these findings often alter the original surgical plan for a wider excision or conversion from BCS the MRM (147). Additionally MRI may alter treatment planning by depicting occult, contralateral BC. In a study at Memorial Sloan-Kettering, MRI detected contralateral BC that was occult mammographically and by physical examination in 5% of women with known BC (148). A similar multi-institutional study found MRI detected contralateral lesions in 3.1% of women with known BC (149). Often these occult lesions are detected in patients with dense breast tissue. While the sensitivity of mammography is inversely proportional to breast tissue density, MRI is extremely useful in this subpopulation, especially since a study by Berg et al demonstrated that bilateral cancer was twice as prevalent in patients with dense breast tissue (150). MRI is also increasingly used to characterize indeterminate findings on mammography. MRI has a negative predictive value (NPV) of 97% to 100% for mammographic abnormalities that lack Gd-DTPA uptake (151).

Limitations of MRI include a high false-positive rate, low sensitivity for detection in situ cancers, and it cannot distinguish between some benign diseases such as fibroadenomas and granulation tissue (10, 143). MRI is useful as an adjunct to mammography and ultrasound in preoperative evaluation of patients with known breast disease as well as for screening of high-risk individuals.

1.6.4 Nuclear medicine imaging

Nuclear medicine imaging is another modality that has been investigated for use in the detection, diagnosis and staging of BC. In contrast to other imaging modalities such as mammography, ultrasound, computed tomography (CT), and MRI which detect mostly anatomical abnormalities of the breast, nuclear medicine imaging uses small concentrations of radioactively-labeled imaging probes (radiopharmaceuticals) to non-invasively detect biological characteristics on a molecular level or sometimes physiological processes of tumours on a molecular level. As such, the term ‘molecular imaging’ is commonly used to describe nuclear medicine imaging techniques. Nuclear medicine imaging is also widely used for the detection and diagnosis of other diseases, including cardiovascular disease, infections and neurological disorders (152). Radiopharmaceutical imaging probes are usually administered intravenously, where they circulate systemically and accumulate in target sites. Patients are then imaged using
dedicated nuclear medicine tomographic cameras to detect gamma-photons emitted from sites of radiopharmaceutical accumulation. Abnormal accumulation of radiopharmaceutical probes may be indicative of malignancy. The sensitivity and specificity of nuclear medicine imaging is dependent on the targeting moiety of the radiopharmaceutical probe, the properties of the radionuclide and the characteristics of the imaging modality. Radiopharmaceuticals are designed either for single-photon emitting radionuclides (e.g. technetium-99m \([^{99m}\text{Tc}]\), indium-111 \([^{111}\text{In}]\) or for iodine-123 \([^{123}\text{I}]\)) or positron emission tomography (PET) using positron-emitting radionuclides (e.g. fluorine-18 \([^{18}\text{F}]\), oxygen-15 \([^{15}\text{O}]\), carbon-11 \([^{11}\text{C}]\), copper-64 \([^{64}\text{Cu}]\), gallium-68 \([^{68}\text{Ga}]\) or iodine-124 \([^{124}\text{I}]\)).

Nuclear medicine can be used to detect both primary and metastatic disease, however due to the limited sensitivity for detection of lesions less than 1 cm in diameter and the radiation absorbed doses associated with radiopharmaceuticals, nuclear medicine is not suitable for screening (153). Currently nuclear medicine is most useful adjunctively for diagnosing clinically palpable or mammographically detectable primary BC lesions. Furthermore, because nuclear medicine imaging often visualizes the whole body, it is useful for detecting local recurrence, as well as distant metastases.

1.6.4.1 Single-photon planar imaging

The gamma camera is the most common imaging device used in nuclear medicine for single-photon planar imaging. A gamma camera generates a two-dimensional image of the distribution and tissue accumulation of imaging probes radiolabeled with single-photon emitting radioisotopes. The optimal energy of gamma rays for imaging with a gamma camera is between 100- and 300 keV. The most frequently used single-photon emitting radioisotopes are \(^{99m}\text{Tc}\), \(^{111}\text{In}\), \(^{67}\text{Ga}\), \(^{123}\text{I}\) and \(^{131}\text{I}\). Following intravenous injection of a radiopharmaceutical, a gamma camera detects photons emitted from a patient using scintillation crystal detectors (often thallium-doped sodium iodide NaI[TI]). Gamma rays interact with the scintillation crystals, producing photons of visible light, which are detected, converted into electrons and amplified by photomultiplier tubes. Collimation of the scintillation crystals has been incorporated to isolate individual gamma photons directly originating from a source in the patient and to reduce processing of scattered or degraded photons, thereby reducing noise and increasing spatial resolution. Collimation, however, attenuates the majority of incoming photons (up to 99.9%)
and greatly reduces the sensitivity of single-photon planar imaging (154). The spatial resolution of planar scintigraphy is governed by the type of collimator, the photon energy, the distance from the source to the detector and the duration of the acquisition (155). More recently, breast-specific gamma imaging (BSGI) cameras have been developed to overcome some of the limitations in resolution and sensitivity of the traditional gamma camera. Sensitivity and resolution are increased, especially for lesions less than 1 cm, by reducing the field-of-view (FoV) and by eliminating the distance between the detector and the patient using compression imaging. Additionally, BSGI cameras have detectors mounted on a flexible gantry arm, allowing replication of standard mammographic views for correlation between these modalities (156).

1.6.4.2 Single-photon emission computed tomography (SPECT)

Single photon emission computed tomography (SPECT) is a nuclear medicine technique for producing 3D cross-sectional images of the distribution of radiopharmaceuticals labeled with single gamma-photon emitting radionuclides. SPECT is performed using a gamma camera fitted with one to three NaI(TI) crystal detector heads which are rotated in small angular steps around the patient. A sufficient number of 2D projections are acquired to generate a tomographic data set. The sensitivity and resolution of SPECT are governed by the same mechanisms as in planar scintigraphy. Following image acquisition, data are reconstructed by filtered back projection or by iterative algorithms to generate a 3D dataset often presented as cross-sectional slices. An advantage of SPECT is that it separates radioactivity distributions that project on top of each other in planar imaging, allowing higher contrast (155). Recently, advances in hardware (e.g. pinhole collimators) and software (e.g. attenuation correction) have improved the sensitivity and spatial resolution of SPECT (154). Current clinical SPECT cameras have spatial resolution of approximately 1 cm full width at half maximum (FWHM), depending on the type of collimator and distance from the source (155, 157). Pinhole collimators can achieve submillimeter resolution in the case of small animal imaging, however pinhole collimation further reduces sensitivity of SPECT imaging (154). Additionally, the development of a SPECT/CT combined imaging modality allows the co-registration of molecular features visualized using radiopharmaceutical probes by SPECT with the precise anatomical depiction provided by CT.
1.6.4.3 Positron-emission tomography (PET)

Positron-emission tomography (PET) is another tomographic nuclear medicine modality that generates 3D data sets of the in vivo distribution of radiopharmaceuticals labeled with positron-emitting radioisotopes. PET detects two coincidence gamma photons with energy of 511 keV simultaneously emitted in virtually opposite directions (angle of \( \approx 180 \) degrees) upon annihilation of a positron by collision with an electron. Most PET cameras are composed of a 360-degree ring of inorganic scintillation crystal detectors (usually bismuth germanate [BGO] or lutetium oxyorthosilicate [LSO]) and affixed photomultiplier tubes (155). The symmetry of the photons allows electronic collimation using a coincidence-timing window of a few nanoseconds to record coincident photon events. This negates the need for physical collimation, improving the sensitivity of PET, which is typically 10-100-fold higher than SPECT (154). Detection of coincidence photons in opposite detectors localizes the annihilation event to a line of response (LOR). During a PET scan several million coincidence events are recorded generating many intersecting LORs providing information on the spatial location of radiopharmaceutical uptake in the body. The improved sensitivity of PET provides better image quality with a reduction in noise. While the spatial resolution of SPECT is limited only by technology, spatial resolution of PET is limited by two intrinsic physical phenomenon, namely positron range and photon non-collinearity. Positron range is the short distance the positron travels before collision with an electron, this distance depends on the energy distribution of the isotope, in which higher energy positrons exhibit a longer range (154). Photon non-collinearity is the slight (0.25 degrees) deviation from 180 degrees between the trajectories of the emitted gamma photons resulting in resolution blurring that depends on the ring diameter of the detector. Current PET scanners have spatial resolution of 4-6 mm FWHM (157). Combined modality PET/CT has received wide clinical acceptance with the obvious advantage of co-registration of the molecular and physiological features imaged by PET with the precise anatomical information provided by CT (154).

Whole body PET using \[^{18}\text{F}]\text{-fluoro-2-deoxy-D-glucose} (^{18}\text{F-FDG})\) has good specificity for BCs, however the intrinsic spatial resolution of the PET technology is poor compared to other radiographic imaging resulting in low detection sensitivity, especially for small lesions (154). Recently, dedicated breast PET (positron-emission mammography) detectors have been
developed. These are small, breast-specific scanners with detectors that are mounted on two compression paddles. Spatial resolution is approximately 1.5 mm FWHM, improving visualization of small lesions (158).

1.7 Nuclear medicine imaging in the detection of breast cancer

Nuclear medicine imaging techniques have been studied for detection of primary BC with indeterminate or mammographically occult lesions. Nuclear medicine imaging may overcome some of the limitations of mammography such as low specificity, high false negative rates for patients with radiographically dense breasts and poor differentiation of recurrent disease and scar tissue following surgery and/or radiotherapy. Various strategies for nuclear medicine imaging of cancer have included tumour perfusion imaging, imaging of glucose metabolism, imaging of cellular proliferation, imaging with monoclonal antibodies or peptides directed against tumour-associated cell surface antigens and receptors, as well as detection of ligands with non-specific uptake. Many of these latter approaches have been used to detect and probe the biological feature of metastatic BC lesions (MBC).

1.7.1 Ligands with non-specific uptake

Nuclear medicine imaging is used in both primary BC and MBC. $^{99m}$Tc-methylene diphosphonate ($^{99m}$Tc-MDP) binds to hydroxyapatite crystals in bone and accumulates avidly in the skeleton, especially in areas of high turnover and increased bone repair (152). Breast cancer bone metastases are visualized by focally increased uptake of $^{99m}$Tc-MDP. Interestingly, while $^{99m}$Tc-MDP has specific uptake in bone, it has also been shown to have non-specific uptake in soft tissue lesions such as BC (152). A variety of factors are thought to contribute to uptake of $^{99m}$Tc-MDP in BC, such as increased vascularity, high calcium content, cell membrane disruption, changes in pH and inflammation. Studies in patients with histologically proven BC showed that $^{99m}$Tc-MDP had a sensitivity of 92% and specificity of 95%; however all lesions smaller than 1 cm were missed by $^{99m}$Tc-MDP imaging (159, 160). False-positives occur in fibroadenomas, acute inflammation and epithelial hyperplasia (153). Similar non-specific uptake of $^{99m}$Tc-diethylenetriaminepentaacetic acid ($^{99m}$Tc-DTPA), a renal imaging agent, has been shown to detect BC with sensitivities and specificities of 75-100% and 91-100%, respectively (153). These agents, however, are not widely used clinically for detection of BC.
1.7.2 Perfusion imaging agents

Perfusion imaging with $^{99m}$Tc-sestamibi (Cardiolite®, Miraluma®, Lantheus Medical Imaging, MA) is the only approved radiopharmaceutical for detection of primary BC imaging (scintimammography) in the U.S. and Canada. $^{99m}$Tc-sestamibi [hexakis(2-methoxyisobutylisonitrile)] was first developed for myocardial perfusion imaging and the first cases of BC detection using the radiopharmaceutical were described in 1992 by Aktolun and co-workers (161). The exact mechanism of $^{99m}$Tc-sestamibi uptake and retention is not fully understood, however it appears to be mediated by increased tumour blood flow and increased cellular uptake and retention by cancer cells. Studies have shown that the uptake of $^{99m}$Tc-sestamibi is associated with increased perfusion of malignant lesions possibly related to the degree of angiogenesis and the percentage of poorly formed blood vessels (162, 163). At a cellular level, studies have shown that 90% of the radiopharmaceutical is accumulated in the mitochondria of tumour cells (164). Sestamibi is a small lipophilic monovalent cation and its uptake is driven by the negative transmembrane potential of the cell and mitochondria (165). Since tumour cells have increased growth and proliferation, they have greater demands for energy and may possess an increased number of mitochondria compared with normal epithelial cells of the breast, leading to greater accumulation of $^{99m}$Tc-sestamibi (165).

The cumulative sensitivity in detecting primary BC from a meta-analysis of published studies with over 3,000 patients was 83% and the average specificity, PPV and NPV were 85%, 85% and 82%, respectively (153). Most of these studies, however, were biased towards larger palpable lesions. The sensitivity for lesions less than 1 cm was low (~50%), and no lesions less than 5 mm were detected (164). Additionally, the benefit of SPECT in detection of primary BC using $^{99m}$Tc-sestamibi is uncertain. Studies comparing SPECT and planar imaging have shown no or modest improvements in sensitivity, but reductions in specificity (153, 164). More recently the introduction of BSGI cameras has improved the sensitivity and resolution for small lesions (156, 166). A retrospective study found that BSGI using $^{99m}$Tc-sestamibi had a sensitivity of 96.4% and a specificity of 59.5%, and correctly identified 16 of 18 breast cancers smaller than 1 cm, with the smallest lesion detected measuring 1 mm (166). Many benign lesions do not avidly accumulate $^{99m}$Tc-sestamibi, however fibroadenomas, abscesses and local
inflammation, mastitis, hamartomas and papillomas commonly result in false positive scans (153).

Aside from its adjunctive use in the detection of BC, $^{99m}$Tc-sestamibi may also be useful in predicting response to chemotherapy. $^{99m}$Tc-sestamibi is a substrate of the transmembrane P-glycoprotein (Pgp-170) which is present in cells overexpressing the multidrug resistance gene (MDRI) and acts as a protective pump to purge potentially harmful molecules out of cancer cells (167). More than 80% of chemotherapeutic agents used in BC are also substrates of Pgp and its expression has been implicated in resistance to chemotherapy in BC (168). Primary BC tumours with high levels of Pgp showed more rapid efflux of $^{99m}$Tc-sestamibi than patients with no or low levels of Pgp (169). Furthermore, $^{99m}$Tc-sestamibi scintimammography in patients with locally advanced BC has shown promise in predicting response and resistance to neo-adjuvant chemotherapy (170). Additionally, an advantage of scintimammography using $^{99m}$Tc-sestamibi compared to conventional mammography is that its sensitivity and specificity seem to be unaffected by breast density or age of the patient. In Canada, $^{99m}$Tc-sestamibi is indicated as a second line diagnostic aid to assist in the evaluation of breast lesions in patients for whom mammography cannot exclude malignancy. It is not, however, indicated for BC screening or as an alternative to biopsy.

Another $^{99m}$Tc-lableled lipophilic monovalent cation, $^{99m}$Tc-tetrofosmin (Myoview, GE Healthcare, U.K.) has also been investigated for perfusion imaging of BC. Similar to $^{99m}$Tc-sestamibi, $^{99m}$Tc-tetrofosmin [1,2-bis(2-ethoxyethyl) phosphino ethane] was originally developed as a myocardial perfusion imaging probe and subsequently approved by the U.S. FDA, but not by Health Canada, for scintimammography of BC. The mechanisms of accumulation in BC are still being investigated, but evidence suggests that both tumour blood flow and Na-K ATPase contribute to tumour uptake (153). Early studies indicate similar sensitivity and specificity to scintimammography $^{99m}$Tc-sestamibi. A meta-analysis of 7 small studies of 400 patients showed that $^{99m}$Tc-tetrofosmin has a mean sensitivity, specificity, PPV and NPV of 94%, 86%, 85% and 91% in lesions greater than 1 cm. More recently BSGI using $^{99m}$Tc-tetrofosmin has improved the detection of non-palpable lesions less than 1 cm. Planar BSGI using $^{99m}$Tc-tetrofosmin was more sensitive with higher specificity than mammography for the detection of primary BC, multifocal and multicentric disease, including lesions less than 1 cm (171, 172).

Perfusion studies have also been performed by PET. Indeed, the first study of BC imaging with PET evaluated tumour blood flow using an [$^{15}$O]oxygen steady-state inhalation
technique (173). In this study, it was found that regional blood flow in tumours was significantly higher than in surrounding normal tissue (173). A later study found that blood flow in BC measured by $[^{15}\text{O}]\text{H}_2\text{O}$ was five-times higher than blood flow in normal tissue, largely due to the higher fat content of normal breast tissue (174). More recently, in a retrospective study tumoural blood flow as measured by $[^{15}\text{O}]\text{H}_2\text{O}$ PET was found to correlate with tumour perfusion measured by DCE-MRI (175). To date, studies of tumour blood flow by $[^{15}\text{O}]\text{H}_2\text{O}$ PET have been restricted to small patient populations and larger, prospective studies are required to more fully describe the utility of $[^{15}\text{O}]\text{H}_2\text{O}$ PET in distinguishing between malignant and normal breast tissue.

1.7.3 Metabolic imaging by PET

PET using $[^{18}\text{F}]-\text{fluoro-2-deoxy-D-glucose (FDG-FDG)}$ has also been explored for imaging BC. $[^{18}\text{F}]-\text{FDG}$ is a glucose analogue that is taken up by cells in proportion to their glucose utilization. PET imaging using $[^{18}\text{F}]-\text{FDG}$ (FDG-PET) is based on the premise that cancer cells have a higher glycolytic rate and higher glucose avidity compared to normal tissue, thus allowing visualization of malignant lesions. $[^{18}\text{F}]-\text{FDG}$ is actively transported into cells by glucose transporters (GLUTs) and is phosphorylated by hexokinases to $[^{18}\text{F}]-\text{FDG-6-phosphate (FDG-6P)}$. Unlike glucose-6-phosphate, $[^{18}\text{F}]-\text{FDG-6P}$ is not a suitable substrate of glucose-6-phosphate isomerase and therefore cannot be converted to fructose-6-phosphate for entry into the glycolytic pathway. Furthermore, $[^{18}\text{F}]-\text{FDG-6P}$ undergoes limited dephosphorylation due to low levels of glucose-6-phosphatase in tumours, thereby becoming metabolically trapped within cells (176). Cancer cells have been shown to overexpress glucose transporters, as well as exhibit increased activity of cytoplasmic hexokinases, which may contribute to the increased uptake and retention of $[^{18}\text{F}]-\text{FDG}$ in malignant cells (177). $[^{18}\text{F}]-\text{FDG}$ accumulation, however, is not specific to tumour cells, inflammation also shows elevated levels of $[^{18}\text{F}]-\text{FDG}$, likely due to uptake by macrophages (176, 178). Additionally, organs with high physiological glucose utilization (e.g. heart and brain) or excretion (e.g. kidneys and liver) also show high normal uptake of $[^{18}\text{F}]-\text{FDG}$ (178).

FDG-PET has been investigated for detection of primary BC, detection of recurrence and metastatic disease, and staging and monitoring of treatment response (see section 1.9.1). Samson et al. reviewed the use of whole body FDG-PET in the detection of primary BC and found that in 13 studies, FDG-PET had a sensitivity of 89%, a specificity of 80% and a negative
predictive value of 87.9%; thus, a false-negative risk of 12.1% (179). Many of these studies, however, were biased towards large palpable lesions. It has also been demonstrated that FDG-PET has poor sensitivity for detecting lobular carcinomas. A study by Kumar et al showed that tumour size less than 1 cm and low tumour grade were significant predictors of false-negative FDG-PET results (180). Primary BC detection requires the ability to demonstrate small (<1 cm) non-palpable, invasive and in situ lesions. Currently, these requirements are beyond the capabilities of whole-body FDG-PET and therefore this modality does not have a clinical role in detecting these small primary BC tumours at this time.

More recently, dedicated positron-emission mammography (PEM) cameras have improved the sensitivity and spatial resolution of PET for detection of BC. A prospective trial blindly evaluated the use of PEM using $^{18}\text{F}$-FDG for the detection of BC in 44 women with confirmed diagnosis of malignancy (181). The average diameter of the tumours was 22 mm and 70% were nonpalpable by clinical examination. PEM detected 89% of the tumours and 3 cases of mammographically occult DCIS were detected by PEM (181). Multifocal disease, however, was correctly identified in only 9 of 14 (64%). All cancer missed by PEM were less than 1 cm, including low- and intermediate-grade carcinomas (181). Other studies have shown sensitivities of 50-90% and specificities of 33-100% (182), however all trials have been small (<90 patients) and more studies are required to define the clinical utility of PEM.

Whole-body FDG-PET adds little value for the detection of primary BC, but FDG-PET has demonstrated usefulness in axillary node and internal mammary node detection in high-risk patients, as well as staging of recurrent and metastatic disease. FDG-PET is recommended for staging patients with recurrent or metastatic disease as it has been shown to be sensitive and specific for these purposes. A study by Moon and co-workers evaluated FDG-PET in patients with suspected recurrence or metastases and found that FDG-PET had a sensitivity of 93% and a specificity of 79% (183, 184). Furthermore, FDG-PET is useful in identifying otherwise occult disease, improving staging accuracy and possibly altering therapeutic strategies. In a study by Eubank et al. FDG-PET altered planned treatment in 44% of patients suspected to have locoregional recurrent BC by demonstrating greater extent of disease than other imaging modalities (184). In another study, FDG-PET/CT had a sensitivity of 90% for diagnosing recurrent BC in asymptomatic BC patients with elevated serum tumour markers (CA15-3, CEA) and resulted in altered clinical management in 51% of the patients (185). Accurate restaging in this setting is extremely important, as therapy for locoregional recurrence may include surgery, radiation, chemotherapy, hormone and biological therapy. FDG-PET is also
valuable in evaluating the extent of disease in distant metastases, but the impact on clinical management is likely to be lower.

1.7.4 Proliferation imaging by PET

Precursors of DNA synthesis such as thymidine analogues have been investigated for imaging tumour proliferation and as a means to overcome the nonspecificity of $^{18}$F-FDG for malignant cells (186). Prior to incorporation into DNA, thymidine is phosphorylated by thymidine kinase and the activity of thymidine kinase generally correlates with DNA synthesis and cellular proliferation (187). A thymidine analogue, 3'-deoxy-3'-[$^{18}$F]-fluorothymidine ($^{18}$F-FLT), is similarly phosphorylated by thymidine kinase, but is not incorporated into DNA and becomes trapped in the cells. Therefore cellular uptake of $^{18}$F-FLT has been proposed as a marker of tumour cell proliferation (186). Indeed, tumour uptake of $^{18}$F-FLT in BC strongly correlates with the Ki67 labeling index, a marker of proliferation, from tumour biopsies (188). A preliminary study of $^{18}$F-FLT for the detection of BC found focally increased uptake of $^{18}$F-FLT in 13 of 14 (92.8%) primary tumours (189). While tumour uptake was lower than that of $^{18}$F-FDG, tumour-to-background contrast was comparable to $^{18}$F-FDG as normal tissue uptake of $^{18}$F-FLT was also lower (189). Preliminary studies have also examined the use of $^{18}$F-FLT for monitoring the response of BC to chemotherapy (see section 1.9.2). Presently, the clinical evidence supporting the use of $^{18}$F-FLT for BC detection remains very limited and larger, prospective studies are required to elucidate the role of $^{18}$F-FLT in the management of BC.

1.7.5 Estrogen receptor imaging by PET

Estrogen receptors (ERs) are an important target for treatment of BC with antiestrogens such as tamoxifen, or aromatase inhibitors such as anastrozole (Arimedex, AstraZeneca, London, UK) and letrozole (Femarals, Novartis, Basel, Switzerland). The presence of ER is the single most important determinant of response to antiestrogen therapy (190). In clinical practice, ER expression is routinely measured by ex vivo IHC of a primary tumour biopsy. This technique is limited due to disease heterogeneity and sampling errors. Furthermore, hormonal therapy is a common first-line treatment for ER-overexpressing MBC, however sampling of metastatic tissue is often difficult, especially in bone, a frequent site of BC metastases. Therefore alternative and complementary methods of measuring ER expression in primary and advanced
BC may improve patient selection for antiestrogen hormone therapy. $^{18}$F-FES (16α-fluoro-17β-estradiol) is a substrate of the estrogen receptor and can be utilized for specific receptor imaging of BC with PET (191, 192). A significant correlation between $^{18}$F-FES uptake in tumours and estrogen receptor expression measured by in vitro assay has been demonstrated and receptor-negative tumours could not be visualized with $^{18}$F-FES (191-193). Additionally, $^{18}$F-FES provided information on the distribution and heterogeneity of ER expression in primary and metastatic sites (194). Notably, high tumour uptake of $^{18}$F-FES prior to therapy has been shown to be predictive of a response to hormonal therapy in advanced BC (192, 195-197). ER blockade following hormone therapy has also been evaluated as a means to monitor and predict response to hormone therapies (see section 1.9.3.1).

### 1.7.6 Antibodies directed against tumour cell surface antigens

Molecular imaging of BC has also been investigated using radiolabeled antibodies (radioimmunodetection) directed against tumour cell antigens and receptors. An advantage of this approach over perfusion and metabolic imaging, is an increased specificity for detecting malignant tissue. Radiolabeled antibodies that bind with high affinity and high specificity to antigens and receptors that are expressed exclusively on cancer cells or at densities that greatly exceed their expression on normal cells are useful molecular markers for distinguishing malignant lesions.

The first clinical evidence of BC imaging using radiolabeled antibodies occurred in 1978 when Goldenberg and co-workers reported detection of an $^{131}$I-labeled goat IgG against carcinoembryonic antigen (CEA) (198). CEA was first thought to be a marker for CRC, however, subsequent studies have shown CEA is expressed in a variety of human carcinomas including 70-90% of BCs as measured by IHC, and serum CEA is elevated in 30-50% of patients with symptomatic MBC (199, 200). Imaging studies using intact anti-CEA antibodies and antibody fragments radiolabeled with $^{131}$I, $^{111}$In or $^{99m}$Tc have all confirmed localization of BC (201). A study by Lind et al. evaluated the use of SPECT with a $^{99m}$Tc-labeled anti-CEA monoclonal antibody in 46 women with suspected BC or recurrence. They found a detection sensitivity of 83% with a specificity of 69%, including 5 false-negative and 5 false-positive results (200). Interestingly, A prospective trial evaluating $^{99m}$Tc-labeled arcitumomab (CEA-Scan®, Immunomedics Inc., Morris Plains, NJ), a Fab fragment of muMAb IMMU-4 in patients with clinically documented (n=49) or suspected BC (n=103) had an overall sensitivity, specificity, PPV and NPV of 61%, 91%, 71% and 86%, respectively. It should be noted,
however, that the sensitivity for nonpalpable disease was low (36%) and 11 of 14 tumours smaller than 1 cm were undetected (202). A second study evaluating $^{99m}$Tc-arctiumomab in 32 high-risk patients (31 with palpable disease) found a reduced sensitivity of $^{99m}$Tc-arctiumomab (77%) compared to $^{99m}$Tc-sestamibi (90%) and no significant improvement in the PPV compared to mammography alone (96% vs. 94%, respectively) (203).

Antibody imaging of BC has also been performed using antibodies against tumour-associated glycoprotein (TAG-72), an epithelial mucin which is overexpressed in 80% of BCs and associated with a more aggressive tumour phenotype (204). In a phase I study all primary tumours, ranging in size from 1.2 cm to 2.5 cm, were detected by imaging using indium-111 labeled antibody B72.3 in 16 patients with known primary BC, however all axillary lymph node metastases were missed (205).

Many different radiolabeled antibodies against MUC1, also known as polymorphic epithelial mucin, have been evaluated for radioimmunodetection of BC (153, 201). MUC1 is a transmembrane high molecular weight glycoprotein which is overexpressed in more than 75% of BCs and this overexpression has been associated with poor prognosis (206). In general, the sensitivity and specificity of anti-MUC1 antibodies for the detection of primary and MBC were modest, ranging from 50-90% (153, 201).

1.7.7 Functional imaging of membrane receptors

Somatostatin receptors (SSTRs) are structurally related integral membrane glycoproteins. To date, five different variants (SSTR1-5) have been identified and approximately 50-75% of primary BCs are SSTR-positive, primarily expressing SSTR2 (207). Somatostatin analogues such as octreotide and pentetreotide have been investigated as a means for detecting primary and metastatic BC. Early studies have shown that approximately 75% of BCs are detected by SSTR scintigraphy using $^{111}$In-pentetreotide and $^{111}$In-octreotide and the detection sensitivity was correlated with lesion size as significantly greater T2 tumours were detected compared to T1 tumours (208, 209). More recently $^{111}$In-pentetreotide was shown to be 80% sensitive for metastases in patients with advanced BC, particularly for bone and lung metastases (210). Furthermore a recent study comparing $^{99m}$Tc-octreotide and $^{99m}$Tc-sestamibi in 12 patients found that the two radiopharmaceuticals had comparable sensitivities for detecting primary BC, but $^{99m}$Tc-sestamibi had higher specificity, PPV and NPV (207). In general, the sensitivity of SSTR
imaging is lower than perfusion agents such as \(^{99m}\text{Tc}\)-sestamibi for the detection of primary BC (153).

Recently, functional imaging of epidermal growth factor family receptors such as EGFR (HER1) and HER2 has been evaluated for predictive and prognostic significance in BC. The EGFR receptor family has a known role in signal transduction controlling normal cell growth, differentiation and survival (31). Dysregulation of these signaling pathways through receptor mutation and/or overexpression has been implicated in breast carcinogenesis and is associated with an aggressive disease phenotype and poor prognosis (26, 211). Strategies for imaging the expression of EGFR family members illustrate the potential role of molecular imaging in personalized cancer medicine. Numerous mAbs and small molecule tyrosine kinase inhibitors (TKI) have been developed to block the mitogenic signaling cascade initiated by the EGFR and its family members. Presently, trastuzumab and lapatinib (Tykerb®, GlaxoSmithKline, U.K.), a pan erbB TKI are the only FDA-approved anti-EGFR therapies for BC, however multiple antibodies and small molecule TKIs have been approved for CRC and NSCLC, including cetuximab (Erbitux, Imclone Systems, New York, NY), matuzumab, panituzumab (Vextibix, Amgen Inc., Thousand Oaks, CA), erlotinib (Tarceva, Genentech Inc., San Francisco, CA), and gefitinib (Iressa, Astrazeneca, U.K.). To date, few studies have evaluated the molecular imaging of EGFR in BC. A preclinical study by our group revealed 10-fold greater tumour localization of \(^{111}\text{In}\)-DTPA-mAb 528, an anti-EGFR antibody, compared to \(^{111}\text{In}\)-labeled human epidermal growth factor (\(^{111}\text{In}\)-hEGF) in human BC xenografts in athymic mice, likely due to the larger size of the antibody leading to increased circulation time and greater accumulation in the tumour (212). In a phase I clinical trial, SPECT imaging using \(^{111}\text{In}\)-hEGF, a radiolabeled form of the natural ligand for the receptor, successfully visualized EGFR positivity in patients with MBC and tumour uptake of \(^{111}\text{In}\)-hEGF correlated with EGFR expression assessed by IHC (213).

Preclinical imaging studies of EGFR expression in xenograft models of other malignancies have also been performed. PET and SPECT studies have evaluated the use of cetuximab radiolabeled with \(^{99m}\text{Tc}\) (214), \(^{111}\text{In}\) (215), \(^{64}\text{Cu}\) (216, 217), and \(^{89}\text{Zr}\) (218) for in vivo detection of EGFR. Interestingly, Cai et al. found a modest correlation between tumour uptake \(^{64}\text{Cu}\)-cetuximab and EGFR expression measured by Western blot, however, a second study found a disparity between tumour uptake of \(^{89}\text{Zr}\)-cetuximab and EGFR expression, indicating that other physiological factors may influence tumour uptake of the antibody. Affibodies and numerous small molecule inhibitors of EGFR have been radiolabeled and evaluated in vitro and in tumour xenograft models with varying success [reviewed by Mishani (219)].
1.8 Approaches to imaging HER2 overexpression using nuclear medicine techniques

Due to its role in the carcinogenesis and progression of BC, HER2 has been studied extensively as a target for anti-cancer therapy (220). The clinical efficacy of many anti-cancer therapies, such as trastuzumab (Herceptin®), rely on receptor overexpression. Therefore, a means to accurately determine HER2 expression levels in individual patients is necessary in order to select those patients who are most likely to respond to treatment. Currently, patients are selected for trastuzumab-based therapy by IHC and/or FISH analysis of a primary tumour biopsy. These techniques, however, are limited by sampling errors due to disease and tumour heterogeneity and some minor discordance between primary tumours and metastases (221). Therefore, the ability to non-invasively quantify or assess total HER2 expression in situ using molecular imaging may provide a means to more accurately select patients for HER2-targeted therapies. Additionally non-invasive imaging of HER2 could be valuable for improving lesion detection, development of new drugs, dose optimization and treatment monitoring (see section 1.9.3.2).

1.8.1 Antibodies and antibody fragments

Clinically, few studies have evaluated the use of non-invasive molecular imaging of HER2. One strategy for imaging of HER2 is the use of radiolabeled mAbs directed against the receptor. The first clinical study in this regard was reported by Behr et al who hypothesized that the therapeutic efficacy and cardiotoxicity of trastuzumab may be related to HER2-mediated specific uptake of the drug in the tumour and heart, respectively, and that this uptake may be visualized by indium-111 labeled trastuzumab ($^{111}$In-trastuzumab) by SPECT imaging (222). The study showed that the anti-tumour efficacy and occasional cardiotoxicity of trastuzumab could be accurately predicted by SPECT imaging using $^{111}$In-trastuzumab. Of 20 patients with HER2-positive BC, all 11 with evidence of tumour uptake had objective responses to the drug. In contrast, only one patient without tumour uptake of $^{111}$In-trastuzumab responded. Moreover, 6 of 7 patients with myocardial uptake of $^{111}$In-trastuzumab subsequently developed cardiotoxicity, whereas none of 13 patients without heart uptake on images exhibited this side effect (222). More recently, however, Perik and co-workers demonstrated that the uptake of $^{111}$In-trastuzumab was not valuable in predicting trastuzumab-mediated cardiotoxicity, but could identify HER2-positive lesions in patients with MBC who had prior anthracycline therapy (223).
In this study, 4 of 15 patients developed severe cardiac dysfunction, but only 1 of these had myocardial uptake of $^{111}$In-trastuzumab (223). In contrast, one or more malignant lesions were visualized in 14 of 15 patients, with 45% of all known lesions detected by $^{111}$In-trastuzumab imaging. Furthermore, previously occult lesions were found in 13 of 15 patients (223), indicating that $^{111}$In-trastuzumab may be complementary to other imaging modalities in determining the extent of disease in patients with HER2-positive BC. Sensitivity of detecting HER2-positive lesions using radiolabeled antibodies may be improved by taking advantage of the 10-100 fold higher photon detection sensitivity of PET. Indeed, early reports of a clinical trial evaluating the use of zirconium-89 labeled trastuzumab ($^{89}$Zr-trastuzumab) in patients with HER2-positive MBC showed excellent tumour uptake and greatly improved resolution compared to $^{111}$In-trastuzumab (224). Furthermore, the Memorial Sloan Kettering Cancer Centre is currently recruiting for a phase I clinical trial evaluating the use of copper-64 labeled trastuzumab ($^{64}$Cu-trastuzumab) in the detection of HER2-positive MBC treated currently with or about to commence treatment with trastuzumab (225). In addition to the improved sensitivity of PET, improvements in the detection of HER2-positive BC for patients undergoing trastuzumab therapy may be achieved using a MAb, antibody fragment, or engineered antibody construct which binds to an epitope distinct from that recognized by trastuzumab. Imaging using radiolabeled trastuzumab during trastuzumab therapy may result in lower sensitivity due to competitive binding of the therapeutic antibodies, resulting in either HER2-receptor blocking or HER2-downregulation, thus reducing the availability of the imaging target. Indeed, Perik et al found that patients undergoing serial scanning during trastuzumab treatment, had lower uptake on their second scan (223). In this thesis, I report the development of a novel HER2 imaging agent consisting of indium-111 labeled pertuzumab ($^{111}$In-DTPA-pertuzumab) which binds to a different epitope of HER2 than trastuzumab and therefore can distinguish between trastuzumab-mediated receptor blocking and receptor downregulation, as well as detect HER2-positive tumours in the presence of trastuzumab occupancy of the receptors.

While clinical reports of HER2 imaging are limited, extensive preclinical studies have evaluated a wide range of HER2 imaging strategies. The first preclinical scintigraphy of HER2 expression was reported by Saga et al, who demonstrated improved targeting and retention of $^{111}$In-labeled anti-HER2 antibody ($^{111}$In-SV2-61r) compared to the same antibody labeled with $^{125}$I ($^{125}$I-SV2-61r), due to the residualizing nature of radiometals (226). $^{111}$In-SV2-61r reached a maximum uptake of 15.3 %ID/g at 48 h post-injection (p.i.) and a tumour-to-blood (T/B) ratio of 5.6, while $^{125}$I-SV2-61r had a T/B ratio of approximately 1.1 at this same time point.
Since this initial study, which demonstrated the feasibility of radioimmunodetection of HER2, antibody imaging has also been performed using a variety of anti-HER antibodies including trastuzumab labeled with single-photon emitters such as $^{111}$In (227-229), $^{131}$I (109, 230) and $^{177}$Lu (231) and positron emitters such as $^{86}$Y (227, 232), $^{64}$Cu (233, 234) $^{124}$I (235) and $^{89}$Zr (236). Most studies demonstrated excellent tumour uptake in HER2-overexpressing xenografts ranging from 12.6 to 66.9 %ID/g and T/B ratios of 1.15 to 6.6 at time-points from 24 to 144 h.p.i. In general, these studies demonstrated superior tumour accumulation and retention of radiometals compared to halogens and the long circulation time of antibodies allowed for excellent tumour accumulation. Prolonged circulation, however, can also result in higher circulating blood radioactivity resulting in modest T/B ratios at earlier time-points. Additionally, the use radiolabeled control irrelevant antibodies and HER2-negative xenografts has shown nonspecific accumulation of radiolabeled antibodies in tumours, likely due to the enhanced permeability and retention effect (EPR), however this was considerably less than the specific uptake of HER-positive xenografts (226, 228, 235).

One strategy to improve the rate of clearance and improve T/B ratios at earlier time-points is to reduce the size of the antibody (~150 kDa) by enzymatic digestion to generate divalent F(ab′)$_2$ fragments (~110 KDa) or monovalent Fab fragments (~55 kDa) (237). Smith-Jones et al demonstrated faster blood clearance and higher T/B ratios of $^{111}$In-labeled trastuzumab F(ab′)$_2$ compared to the intact antibody at 24 h p.i. in BT-474 human BC xenografts (10.0 vs. 3.4, respectively, calculated from the presented data) (234). In this same study, high contrast PET images of BT-474 human BC xenografts were achieved after only 3 h following injection of $^{68}$Ga-labeled trastuzumab F(ab′)$_2$ fragments (234). Tang and co-workers also demonstrated improved T/B ratios of $^{111}$In-labeled trastuzumab Fab fragments, achieving T/B ratios of 25:1 at 72 h.p.i. At 24 h, however, T/B ratios of $^{111}$In-trastuzumab Fab and $^{99m}$Tc-trastuzumab Fab were only 4.2 and 3.2, respectively, lower than that achieved by the $^{68}$Ga-trastuzumab F(ab′)$_2$, possibly due to reduced avidity of the monovalent Fab (234, 238, 239). These studies also reported increased kidney uptake (>50 %ID/g at 24 h p.i.) compared to full-length antibodies, a frequent observation of imaging probes smaller than 60 kDa (234, 238, 239). To modulate clearance from the kidneys and improve tumour localization, an anti-HER2
4D5 Fab has been fused with an albumin-binding peptide (AB.Fab4D5) and labeled with $^{111}$In to generate a bifunctional imaging probe capable of binding albumin and HER2 (240). Association with albumin improved tumour localization by increasing tumour retention compared to $^{111}$In-Fab4D5 and improved distribution homogeneity compared to intact 4D5 (240). Tumour uptake was comparable to the intact antibody at 48 h p.i. (35.9 vs. 38.2 %ID/g, respectively), however $^{111}$In-AB.Fab4D5 accumulated in tumours more quickly and had faster elimination from the blood. Importantly, however, despite the faster clearance, $^{111}$In-AB.Fab4D5 did not accumulate in the kidney, suggesting that association with albumin leads to an altered route of elimination and catabolism (240).

1.8.2 Engineered antibody radioimmunoconjugates

Engineered antibody constructs offer a promising strategy to improve blood clearance and target avidity in designing optimal imaging probes (241). A single-chain Fv (scFv; ~25 kDa) recombinant protein can be engineered by connecting the genes for the heavy and light-chain variable regions by an oligonucleotide linker, which when expressed in a suitable host cell forms a single polypeptide chain with a linker sequence bridging the two variable domains (242). Dimers of scFv’s can form either by spontaneous association of monovalent scFv’s (diabodies; ~55 kDa) or by covalently coupling 2 scFv’s with a peptide linker [sc(Fv)$_2$; ~60 kDa]. Larger still are minibodies, which consist of scFv’s fused to single constant domains of IgGs; ~80 kDa). The different sizes, affinities and valencies of the various constructs results in different clearance rates and mechanisms as well as different tumour-targeting abilities (241). Numerous such engineered antibody molecules have been evaluated for non-invasive imaging of HER2.

Due to their small size, scFvs have faster blood clearance compared to IgGs, Fab or F(ab’)$_2$ fragments and better tumour penetration (243). However, due to their small size and monovalent nature, scFvs have suboptimal tumour accumulation (243). The formation of scFv dimers can improve tumour uptake by prolonging blood clearance and improving bivalent avidity (243). An elegant study by Adams and co-workers demonstrated that the increased avidity of bivalent scFv dimers is a greater contributor to their improved tumour localization and retention compared to their increased size and slower clearance kinetics (244). This was demonstrated by comparing the tumour uptake of HER2-divalent 741F8-1 (scFv)$_2$ homodimers with monovalent 741F8/26-10 (scFv)$_2$ heterodimers with specificity for HER2 (741F8) and
digoxin (26-10), and with the monovalent 741F8-1 scFv monomer (244). Tumour localization in SCID mice bearing SKOV-3 HER2-overexpressing ovarian cancer xenografts at 24 h p.i. was 3.57 %ID/g for the $^{125}$I-741F8-1 (scFv)$_2$ compared to 1.13 %ID/g for the $^{125}$I-741F8/26-10 (scFv)$_2$ and 1.25 %ID/g for the $^{125}$I-741F8-1 scFv monomer. Similarly, the T/B ratio at 24 h p.i. was greater for the divalent dimer (19.1) compared to the monovalent dimer (7.3) or the scFv monomer (10.6). Of the two dimers (both 54 kDa), the divalent construct exhibited significantly greater tumour accumulation compared to the monovalent dimer, which had tumour accumulation similar to that of the smaller scFv monomer (27 kDa) indicating the greater influence of avidity compared to clearance kinetics (244). Other studies have confirmed the improved tumour localization and imaging properties of scFv dimers compared to scFv monomers and Fab fragments (245-247). Non-covalent scFv dimers (diabodies) have also been shown to improve tumour retention over scFv monomers (248). PET studies of $^{124}$I-labeled C6.5 diabodies ($^{124}$I-C6.5) demonstrated that tumours could be visualized at 4 h p.i., however the best contrast was achieved at later time-points with a T/B ratio of 23.4 at 48 h p.i (249). While scFv dimers and diabodies have shown greater tumour accumulation and retention than scFv monomers, affinity and valency are not the only factors involved in improved tumour uptake. Indeed, Adams et al have shown that while a minimum affinity (10$^{-8}$ M) is required for adequate tumour accumulation, affinities above 10$^{-9}$ M show no improvement in tumour localization. In contrast, affinities above 10$^{-9}$ M resulted in increased blood concentrations and lower T/B ratios of scFv’s (250). The authors propose that impaired tumour penetration of high affinity molecules limits their overall specific tumour uptake (250). Therefore, it is possible that slower clearance, dictated by larger molecules may be important for further improvements in tumour uptake of engineered antibody constructs.

Minibodies are a promising approach, which retain the greater avidity of divalent constructs while their larger size results in longer blood circulation. Both hinged and hingeless minibodies have been constructed by fusing a scFv to a human IgG1 C$_{H}$3 domain. With an intermediate molecular weight of ~ 80 kDa, less than that of F(ab’)$_2$ fragments, minibodies allow for greater accumulation in the tumour, while still clearing rapidly resulting in good T/B ratios at early time-points (243). Minibodies were first developed against CEA and demonstrated excellent tumour targeting properties in mice bearing LS174T human CRC xenografts (251).
123I-labeled minibodies demonstrated rapid, high tumour uptake, reaching up to 33 %ID/g at 6 h p.i. and T/B ratio of 64.9 at 48 h p.i., permitting imaging of xenografts only 4 h following administration (251). HER2 minibodies with high affinity (2-4 nM) have been engineered from the parental internalizing 10H8 anti-HER2 antibody and labeled with 131I for evaluation in biodistribution studies in athymic mice bearing MCF7/HER2 xenografts (252). Although T/B ratios exceeded 3:1 at 48 h p.i., tumour uptake reached a maximum of 5.6 %ID/g at 12 h p.i. and was reduced to 2.0 %ID/g at 48 h p.i. (252). Dehalogenation following internalization of the minibody is one possible explanation for the reduced tumour uptake, however further studies by Olafsen and co-workers demonstrated that tumour uptake of 111In-labeled 10H8 (111In-10H8) minibodies had a similar maximum tumour uptake of 5.7 %ID/g at 24 h p.i., but a much improved T/B ratio of 13.3 at 48 h p.i. (253). Furthermore, a 64Cu-labeled minibody derived from trastuzumab (64Cu-hu4D5v8) was compared with a larger construct [(scFv-C12-C13)2; 105 kDa] engineered to minimize interaction with neonatal Fc receptor (FcRn), and therefore retain circulation clearance similar to that of smaller constructs (~80 kDa). While tumour uptake of 64Cu-hu4D5v8 was similar to that of 111In-10H8 minibodies, the larger (scFv-C12-C13)2 construct had higher tumour accumulation (12.2 %ID/g) in HER2-overexpressing xenografts at 21 h p.i., allowing discrimination between low-HER2 expressing xenografts (T/B ratio of 3.4) and high HER2-expressing xenografts (T/B ratio of 4.5) on microPET images (253). Interestingly, the larger (scFv-C12-C13)2 construct also demonstrated lower kidney uptake than the HER2 minibodies, which have elevated kidney uptake due to a cross reactive antigen in the kidneys (253).

These studies demonstrate that engineering antibody constructs with varying sizes, valencies, affinities and radiolabeling techniques can result in significant differences in distribution and tumour accumulation, allowing manipulation of pharmacokinetic and targeting properties to design optimal imaging probes.

1.8.3 Affibodies

In the last 5 years, a significant body of work has demonstrated the excellent tumour targeting and pharmacokinetic properties of small non-immunoglobulin based imaging agents known as affibodies. Affibody molecules are 58-amino acid in length proteins derived from the domain scaffold of the immunoglobulin-binding staphylococcal protein A. The small size (~7 kDa) of affibody molecules enables fast blood clearance and good tumour penetration (254).
Randomization of 13 amino acid positions in the binding surface of the domain scaffold has generated combinatorial phagemid libraries from which affibody molecules with affinities towards a variety of cell surface antigens can be selected by phage display (254). The first anti-HER2 affibody, ZHER2:4, bound to the ECD of HER2 with 50 nM affinity at a different site than that recognized by trastuzumab (255).

A significant advancement in targeting HER2 using affibody molecules was achieved with the affinity matured second generation affibody, ZHER2:342, which has an affinity of 22 pM for an epitope in the ECD of HER2 distinct from that recognized by trastuzumab (256). When radioiodinated, the high affinity affibody (125I-PIB-ZHER2:342) demonstrated specific tumour accumulation and rapid blood clearance (9.5 %ID/g and T/B of 37.8 at 4 h p.i.) in athymic mice bearing SKOV-3 xenografts, which was 4-fold higher than the previous generation affibody (ZHER2:4) (256). Radiolabeling the affibody with 111In (111In-ZHER2:342), resulted in even greater improvements in the T/B ratio at early time points, due to higher tumour accumulation and faster blood clearance, however higher uptake was also measured in the kidneys (257-259). Overall tumour uptake of these agents was 10-12 %ID/g at 4 h p.i. and T/B ratios of 111In-Bz-DTPA-ZHER2:342 and 111In-CHX-A”-DTPA-ZHER2:342 were >100 at 4 h p.i., significantly higher than any other anti-HER2 imaging probe (257, 259).

Affibodies have also been successfully labeled with positron-emitting isotopes such as 124I (260) and 18F (261, 262) for PET imaging. Kramer-Marek and co-workers labeled ZHER2:342 with 18F by conjugating the affibody with N-2-(4-[18F]fluorobenzamido)ethyl]maleimide ([18F]FBEM-ZHER2:342). Tumour uptake was 9.73 %ID/g at 1 h p.i. and 6.22 %ID/g at 4 h p.i. Rapid blood clearance resulted in T/B ratios of 7.5 at 1 h p.i. and 69 at 4 h p.i. generating high contrast PET images as early as 1 h p.i. (262). Successful imaging has been achieved using 124I-labeled affibodies (260), however an advantage of 18F is its short physical decay half-life which facilitates serial monitoring of receptor status by imaging over a short time frame.

Preliminary clinical data of 111In-DOTA-ZHER2:342 and 68Ga-DOTA-ZHER2:342 affibody molecules for imaging HER2 have been reported at scientific meetings and have demonstrated that affibody-based PET and SPECT imaging agents generate high quality images and can detect small HER2-positive lesions, at least in the small subset of patients evaluated (263, 264).
1.8.4 Peptides

Aside from affibodies, other short binding peptides have also been investigated for targeted therapy and imaging of HER2. A cyclic 20 amino acid peptide, EC-1, has been shown to bind specifically to HER2 overexpressing SKBR-3 cells and inhibit HER2 phosphorylation in a time- and dose-dependent manner (265). This peptide has also been conjugated to green fluorescence protein (GFP) for studying peptide-induced HER2 downregulation *in vitro* in HER2-overexpressing breast and ovarian cancer cells (266). A hexapeptide, KCCYSL, has been shown to bind to HER2-overexpressing MDA-MB-435 cells with micromolar affinity (267) and has recently been radiolabeled with $^{111}$In for imaging of HER2 in MDA-MB-435 xenografts in SCID mice (268). This peptide, $^{111}$In-DOTA-KCCYSL, demonstrated a T/B ratio of 5.1 at 2 h p.i., which corresponded to an absolute tumour uptake of $0.66 \pm 0.11 \%$ID/g (268). Similarly, another HER2 peptide, AHNP, which inhibits the growth of HER2-overexpressing SKBR-3 cells, has recently been radiolabeled with $^{99m}$Tc for detection of HER2 overexpression in T6-17 tumours (NIH3T3 cells transfected to overexpress HER2) (269, 270). Although, $^{99m}$Tc-AHNP demonstrated low tumour uptake ($0.24 \pm 0.05 \%$ID/g) at 3 h p.i., which corresponded to a T/B ratio of only $0.43 \pm 0.15$ (270). In general, results of radiolabeled HER2 peptides have been less successful than affibodies and antibody-based immunoconjugates.

1.8.5 Summary of nuclear medicine techniques for imaging HER2

HER2-specific antibodies, antibody fragments, engineered immunoglobulin constructs and affibodies have all been evaluated for non-invasive imaging of HER2 receptor expression levels. To date, most work has been performed in preclinical models in proof-of-principle studies demonstrating the feasibility of visualizing HER2-positive BC xenografts. Most clinical data has focused on radiolabeled intact antibodies, which have the highest tumour accumulation (%ID/g) of the various imaging agents. The slower blood clearance of full-length antibodies, however, has initiated studies focused on designing high affinity, rapidly clearing HER2 imaging agents. Studies have shown that affinity, valency, size, isotope, and method of radiolabeling can all significantly affect the tumour uptake and blood clearance of imaging probes. Presently, affibodies have demonstrated the highest T/B ratios at the earliest time points, allowing high contrast SPECT and PET imaging as early as 1 h after administration. Radiolabeled antibodies, however, are irreplaceable for evaluating the delivery and dosimetry of antibody-based drugs for immuno- and radioimmunotherapy. Preliminary preclinical studies on molecular imaging of HER2 overexpression are promising and warrant further investigation for detection of HER2
positive BC in patients, for predicting and monitoring response to anti-HER2 therapy, as well as
detection of recurrent disease.

1.9 Monitoring response of breast cancer to treatment using radionuclide molecular
imaging

Reliable and standardized methods of evaluating tumour response during and following cancer
treatment are essential for the management and care of patients as well as for clinical research.
The most commonly used guidelines, known as RECIST (response evaluation criteria in solid
tumours), were developed to simplify and standardize monitoring of patient response in clinical
trials of anti-cancer drugs (271). These methods are based on the assessment of the size of
primary tumours and metastases and have been validated in studies for the response to cytotoxic
chemotherapy. It is unknown, however, if these criteria are sufficient or adequately sensitive for
detecting tumour response to targeted cancer therapies, such as trastuzumab. Trastuzumab and
other targeted therapies have multifactorial and complex mechanisms of action (see section 1.3),
often resulting in a combination of cytostatic and cytotoxic effects, which may or may not lead
to a change in tumour size. Nuclear medicine imaging, which utilizes radiopharmaceutical
probes to non-invasively detect the target molecules and physiological processes involved in
tumourigenesis and progression of cancer may offer a sensitive technique to monitor response to
these targeted therapeutics. Furthermore, molecular changes in cell-receptor status, metabolism,
angiogenesis and other processes may occur before anatomical changes are detected by CT and
MRI, possibly allowing an earlier assessment of tumour response to targeted therapies, as well
as chemotherapy and hormone therapy.

1.9.1 Metabolic imaging of response using $^{18}$F-FDG

$^{18}$F-FDG, a glucose analogue which is taken up by cells in proportion to their metabolic rate (i.e.
glucose utilization), is by far the most studied imaging agent for PET (see section 1.7.3) as well
as for nuclear medicine monitoring of the response of BC and other malignancies to therapy
(135, 272-279). Conventional radiologic imaging is not optimal for monitoring response to
treatment because it cannot distinguish viable tumour tissue from fibrotic scar tissue in patients
with residual masses during or after treatment. Furthermore, conventional imaging modalities
rely on changes in tumour volume, which may take many cycles of cytotoxic therapy to
manifest due to the need for tissue remodeling following elimination of killed cancer cells, and may not occur for cytostatic therapies. Therefore, changes in the metabolic rate of tumours may provide an earlier and more accurate indication of subsequent tumour response.

FDG-PET has been used to monitor response to chemotherapy, hormone therapy and biologically targeted therapies. In BC and other malignancies, experience with FDG-PET for biological therapies remains limited, but preliminary studies have evaluated FDG-PET for monitoring response to bevacizumab (274, 279), cetuximab (273), gefitinib (278), lapatinib (276), pertuzumab (135), and imatinib (272, 275, 277).

(i) $^{18}$F-FDG for monitoring chemotherapy of breast cancer

FDG-PET has been evaluated for response to neoadjuvant chemotherapy for LABC as well as for patients with recurrent or metastatic disease. For patients with LABC, serial FDG-PET has been widely studied as a means to predict response to neoadjuvant chemotherapy using comparison to histopathologic assessment following surgery as the “gold” standard. Most studies have evaluated FDG-PET at baseline and either at the midpoint of therapy (280-284) or after the first or second cycle of therapy (282-288). Generally, the majority of studies performed at the midpoint of therapy demonstrate that a decline of approximately 50% in tumour uptake of $^{18}$F-FDG is associated with response to neoadjuvant chemotherapy, compared to a more modest decline or increase in $^{18}$F-FDG uptake non-responding patients (280-284). Additionally, in some cases earlier signs of response can be achieved after only 1 or 2 cycles of chemotherapy (282-288). Rousseau et al recently reported on the use of serial FDG-PET for predicting response to neoadjuvant chemotherapy in 64 patients with stage II and III BC who underwent FDG-PET after the first, second, third and sixth courses of chemotherapy, after which surgery and histopathological analysis was performed. Using a cutoff of a 60% decrease in the baseline standard uptake value (SUV), the sensitivity, specificity, positive predictive value and negative predictive value of FDG PET were 61%, 96%, 95% and 68% after one course of chemotherapy and 89%, 95%, 89% and 85% after two courses and 88%, 73%, 81% and 83% after three courses, respectively, indicating that FDG-PET may predict response to adjuvant chemotherapy most accurately after 2 courses of chemotherapy (288). Similarly, Kumar and co-workers found that FDG-PET has a sensitivity, specificity and accuracy of 93%, 75%, and 87% for predicting response to neoadjuvant chemotherapy in patients with LABC after 2 courses of chemotherapy (286).
Metastatic breast cancer is often responsive to chemotherapy, and while cure is rarely achieved, with optimal therapy patients can experience prolonged survival and improved quality of life. FDG-PET may also be valuable in this setting providing early assessment of response to therapy, allowing oncologists to modify the therapy to maximize benefit and reduce side-effects. Gennari and co-workers studied serial FDG-PET in 9 patients with MBC at baseline and after 1 course of therapy. In this study a decrease in SUV from baseline of ≥ 50% was predictive of a response, whereas there was no significant decrease in the SUV for nonresponding patients (289). These results were confirmed in a study by Schwarz-Dose and co-workers who demonstrated a decline of approximately 50% from the baseline SUV in responding patients and a more modest decline in nonresponders (290). In this study, patients classified as responders (n=6) as assessed by FDG-PET had an average overall survival of 19.2 months compared with nonresponders (n=5) who had an overall survival of 8.8 months (290). For patients with metastatic disease, treatment response assessment of bone metastases can be particularly difficult. A recent study demonstrated that FDG-PET was useful in predicting the response of bone metastases to chemotherapy and that patients with smaller decreases in SUV from baseline had a shorter TTP. In summary, FDG-PET is a promising imaging modality for assessing response in patients with LABC as well as metastatic disease, however, larger prospective trials are required to determine validated cutoff values (% decrease in SUV) to accurately distinguish between responders and nonresponders. An appropriate cutoff value must have a high negative predictive value (NPV) in order to alter therapy based on the FDG-PET assessment.

(ii) $^{18}$F-FDG for monitoring hormone therapy for breast cancer

FDG-PET has also been evaluated for monitoring the early response to hormone therapy in ER-positive MBC. In contrast to the decrease in tumour uptake of $^{18}$F-FDG following chemotherapy, increases in $^{18}$F-FDG soon after initiation of tamoxifen therapy correlate with early response to hormone therapy (196, 197, 291). This increase in tumour uptake known as ‘metabolic flare’ is produced by temporary estrogen-like agonist effects of the drug. Studies in ER-positive MBC have shown evidence of metabolic flare 7-10 days following initiation of tamoxifen therapy corresponding with an increase in tumour uptake of $^{18}$F-FDG only in
responding patients, while nonresponders have no significant changes in tumour uptake of $^{18}$F-FDG (196, 291). A more recent study examined if metabolic flare, assessed by FDG-PET, was also associated with response to other hormone therapies such as aromatase inhibitors or fulvestrant, an estrogen receptor antagonist (197). In this study 51 patients with advanced ER-positive BC underwent FDG-PET at baseline and after administration of 30 mg of estradiol (an ER agonist to stimulate a flare reaction). Patients were then treated with an aromatase inhibitor or fulvestrant and monitored for response. A percent increase in SUV of $\geq 12\%$ was chosen to represent a metabolic flare reaction. Of the 51 patients, only responders ($n=17$) demonstrated a metabolic flare (average percent increase in SUV of $20.9 \pm 24.2\%$), while nonresponders had no increase in $^{18}$F-FDG uptake following estradiol (-4.3 $\pm$ 11.0%; $P<0.0001\%$). Furthermore, metabolic flare was associated with a significantly longer overall survival ($P=0.006$). These studies have demonstrated that early changes in uptake of $^{18}$F-FDG may be predictive of subsequent response or resistance to hormone therapy in advanced ER-positive BC.

(iii) $^{18}$F-FDG for monitoring response to targeted cancer therapies

Response to targeted cancer therapies, particularly directed against HER2, have also been associated with decreased uptake of $^{18}$F-FDG in studies of small groups of patients, heavily pretreated with chemotherapy (135, 276). FDG-PET and CT were performed at baseline and at 1, 2, 3, 5, 7, and 10 months after the initiation of lapatinib, a small molecule pan erbB TKI, in 8 pre-treated patients with various solid tumours, including 1 patient with advanced BC (276). Based on the CT assessment, there was 1 partial response (PR; patient with BC), 4 patients with stable disease (SD) and 3 patients with progressive disease (PD). The patient who had a PR had a 60% decrease in the tumour uptake of $^{18}$F-FDG and those with stable disease had decreases in tumour uptake of 6-42% one month after the start of treatment, while 2 of 3 patients with PD had an increase in the tumour uptake of $^{18}$F-FDG (276). The patient with SD who had the greatest decrease in tumour uptake of $^{18}$F-FDG (42%) experienced the longest duration of stable disease (10 months) compared to those with smaller decreases in tumour uptake (3-4 months). Furthermore, the SUV$_{\text{MAX}}$ of the patients with prolonged SD and PR began to increase 1-2 months earlier than documented disease progression by CT, indicating that monitoring therapy with FDG-PET may provide an earlier indication of response and/or emergence of resistance to lapatinib therapy (276).
Response to treatment with pertuzumab, a mAb dimerization inhibitor of HER2, has also been evaluated by FDG-PET in an exploratory study in 22 patients with NSCLC (135). In this study, tumour response by CT was assessed at 6, 12 weeks and every 3 months thereafter. Exploratory PET was performed at baseline and 29-42 days after the initiation of treatment. Of the 22 patients, 6 patients (27%) had an average decrease in tumour uptake of $^{18}$F-FDG assessed by $SUV_{MAX}$ of $\geq 25\%$, which is consistent with European Organization for Research and Treatment of Cancer (EORTC) criteria for a partial metabolic response (292). Patients with a partial metabolic response (n=6) had prolonged progression free survival (35.4 weeks) compared to those without a metabolic response (6.1 weeks; P=0.018), demonstrating promising results for the use of FDG-PET in monitoring response to targeted HER2 therapy (135).

FDG-PET has also been explored with promising results in monitoring response to a variety of targeted therapies directed against EGFR, VEGF, BCR-ABL and VEGFR for malignancies other than BC. To date, no clinical studies have evaluated the use of FDG-PET for identifying response or resistance to trastuzumab (Herceptin®) in BC. In this thesis, I have investigated if changes in the tumour uptake of $^{18}$F-FDG, assessed by microPET, correlate with response to trastuzumab in human BC xenografts in athymic mice (Chapter 4). This study provides promising results for an upcoming clinical trial currently recruiting patients for the use of FDG-PET in advanced BC patients treated with trastuzumab or receiving hormone therapy (293).

1.9.2 Imaging proliferation using $^{18}$F-FLT to monitor response to treatment

PET imaging using $^{18}$F-FLT (3'-deoxy-3'-$[^{18}\text{F}]-\text{fluorothymidine}$) has also been investigated as a marker of BC response following chemotherapy (294-297). Conflicting preclinical data indicate that differing mechanisms of action of chemotherapeutic agents may have distinctive effects on the uptake of $^{18}$F-FLT. An in vitro study by Direcks et al using MDA-MB-231 human BC cells demonstrated a 54% decline in cellular uptake of $^{18}$F-FLT following 72 h of treatment with 5-fluorouracil (5-FU), but a paradoxical 173% increase following treatment with doxorubicin and no change in the cellular uptake of $^{18}$F-FLT following treatment with paclitaxel (294). In contrast, a study using MCF-7 human BC cells found that both doxorubicin and docetaxel resulted in decreased uptake of $^{18}$F-FLT and the extent of uptake reduction correlated with the concentration of the chemotherapeutic agent and a reduction in cellular proliferation (295).
Preliminary clinical data indicate that $^{18}$F-FLT imaging may be a sensitive probe of early changes in cellular proliferation following chemotherapy (296). Thirteen patients with stage II-IV BC were treated with a combination of 5-FU, epirubicin and cyclophosphamide (FEC regimen) and $^{18}$F-FLT uptake was measured prior to therapy and at 1-week post-treatment. A reduction in tumour uptake (SUV) of $^{18}$F-FLT discriminated between patients with clinical response and stable disease ($P=0.02$) (296). Of the 13 patients, all 6 with a clinical response had a significant reduction in $^{18}$F-FLT uptake. Another small study evaluated the use of $^{18}$F-FLT PET in predicting response to chemotherapy in 14 patients with newly diagnosed primary or MBC (297). Patients were scanned prior to treatment, as well as 2 weeks following the end of the first cycle and again following the final cycle or 1-year after the initiation of treatment, which ever came first. The study demonstrated that mean changes in $^{18}$F-FLT uptake (SUV) in primary and metastatic tumours after the first course of therapy correlated with late changes in tumour size measured by CT as well as tumour response ($r=0.74$, $P=0.01$) (297). To date, no large prospective clinical trials have evaluated the use of $^{18}$F-FLT in predicting response to chemotherapy in BC.

1.9.3 Imaging changes in growth factor receptor availability to monitor response

Targeted anti-cancer therapy relies on the efficient delivery of drugs to tumour cells which interact specifically with the molecular targets. In the case of hormone and peptide growth factor receptors, treatment could result in a reduced availability of the receptor either by blocking or by downregulation (i.e. internalization and degradation of the receptor). The ability to measure the extent of receptor-blocking and/or downregulation may therefore provide information of the effectiveness of drug delivery to tumours as well as report on their efficacy in individual patients, especially for those agents in which these processes are intimately involved in their mechanism of action.

1.9.3.1 Estrogen receptors (ER)

PET imaging studies measuring pre-treatment uptake of $^{18}$F-FES (16$\alpha$-fluoro-17$\beta$-estradiol), a substrate of the estrogen receptor, have shown its utility in selecting patients for hormone therapy (see section 1.7.5). Interestingly, a reduction in tumour uptake of $^{18}$F-FES following treatment with tamoxifen correlates with ER-blocking by the drug and has been shown to be an early marker of response to hormone therapy. Studies in patients with ER-positive MBC imaged before and 7-10 days following initiation of tamoxifen therapy have shown that the magnitude
of ER blockade by tamoxifen is significantly greater in responders compared to non-responders (196, 291). Furthermore, higher baseline uptake of $^{18}$F-FES correlates with response to tamoxifen, aromatase inhibitors and fulvestrant since it likely reflects a higher level of ER expression (196, 197, 291).

A single-photon emitting imaging agent $^{123}$I-labeled cis-11β-methoxy-17α-iodovinylestradiol (Z-$^{123}$I-MIVE) has been evaluated in 23 patients with ER-positive MBC (298). Scintigraphy was performed prior to and 4 weeks following initiation of tamoxifen therapy. At baseline, 21 of 23 patients had high tumour uptake of Z-$^{123}$I-MIVE, with the remaining two patients showing weak tumour uptake. After initiation of tamoxifen therapy, 17 of the 21 patients with high baseline uptake had complete ER blockade, the remaining 4 patients showed moderate or no blockade. After follow-up it was shown that none of the patients with low initial Z-$^{123}$I-MIVE uptake (n=2) or patients with moderate or no blockade (n=4) responded to treatment, while all 17 patients with high baseline uptake and complete ER blockade had significantly longer progression-free survival (298). These studies demonstrate that ER occupancy characterized in vivo by PET or gamma scintigraphy provides evidence of adequate delivery of the drug and may be predictive of response to tamoxifen therapy in advanced BC.

1.9.3.2 HER2

HER2 downregulation is one of the proposed mechanisms of action of trastuzumab and decreased surface expression of HER2 has been proposed as the mechanism for other targeted anticancer therapies such as heat shock protein-90 (Hsp90) inhibitors. Hsp90 inhibitors, such as geldanamycin and its derivatives, inhibit HER2 signaling by inducing HER2 proteasomal degradation, resulting in a reduction in membrane receptor density (234). These agents induce protein degradation by binding to and inhibiting the function of the Hsp90 chaperone protein, which is required for the conformational maturation and stability of a variety of signaling proteins, including HER2. Hsp90 inhibitors have shown anti-tumour efficacy in preclinical human tumour xenograft mouse models and are currently under clinical evaluation for treatment of HER2-overexpressing BC (299). Smith-Jones et al were the first to demonstrate that changes in HER2 density, mediated by the Hsp90 inhibitor 17-AAG (17-allylaminogeldanamycin), could be quantitatively measured by PET imaging using $^{68}$Ga-DOTA-conjugated Herceptin (trastuzumab) F(ab')$_2$ fragments ($^{68}$Ga-DCHF) (234). $^{68}$Ga-DCHF specifically accumulated in
BT-474 HER2-overexpressing BC xenografts in athymic mice and high-contrast PET images could be acquired as early as 3 h following administration of the radiopharmaceutical. Twenty-four hours following treatment with 17-AAG, there was an 80% reduction in the specific tumour accumulation of $^{68}$Ga-DCHF measured by volume-of-interest (VOI) analysis of PET images and confirmed by biodistribution studies (234). The reduction in tumour accumulation of $^{68}$Ga-DCHF correlated well with *ex vivo* Western blot analysis of lysed tumour tissue, which indicated a similar 80% reduction in HER2 expression (234).

$^{68}$Ga-DCHF is not suitable, however, for monitoring changes in HER2 density as a result of trastuzumab therapy. $^{68}$Ga-labeled trastuzumab F(ab$^\prime$)$_2$ would bind to the same epitope as the unlabeled therapeutic trastuzumab and would be unable to distinguish between receptor downregulation and receptor blocking. Instead, imaging probes that bind to an epitope distinct from that recognized by trastuzumab will be required for monitoring trastuzumab-mediated HER2 receptor downregulation.

Recently, Kramer-Marek and co-workers developed and studied a fluorine-18 labeled affibody ($^{18}$F-FBEM-Z$_{HER2:342}$) which binds to HER2 at a different epitope than trastuzumab, for monitoring the response of BC xenografts in mice to Hsp-90 inhibitors. In this study, PET imaging with $^{18}$F-FBEM-Z$_{HER2:342}$ sensitively detected HER2 downregulation following treatment with the Hsp90 inhibitor 17-demethoxygeldanamycin (17-DMAG) (300). Treatment with 17-DMAG resulted in a 70% reduction in the tumour uptake of $^{18}$F-FBEM-Z$_{HER2:342}$ in athymic mice bearing BT-474 xenografts, which correlated well with reductions in HER2 receptor density measured *ex vivo* by ELISA and Western blot. Interestingly, however, there was no change in HER2 density measured *ex vivo* by IHC, indicating that IHC was insufficiently sensitive in this study to detect these changes in HER2 expression (300). No correlations were made, however, with tumour response to 17-DMAG or to different doses of this agent.

To date, there have been no reports of radionuclide molecular imaging of trastuzumab-mediated HER2 downregulation, however, a biodistribution study by Cheng et al reported a 2-fold reduction in the tumour uptake of fluorine-18 labeled affibody ($^{18}$F-FBO-Z$_{HER2:477}$) following pre-treatment of mice with SKOV-3 xenografts with a single dose of trastuzumab (261). In this thesis, I describe my investigations of the potential of $^{111}$In-labeled pertuzumab ($^{111}$In-DTPA-pertuzumab) to probe trastuzumab-mediated HER2 downregulation by microSPECT/CT imaging in athymic mice bearing HER2-overexpressing BC xenografts.
1.9.4 Imaging molecular markers of tumour resistance

Alternatively, insight into tumour response may also be gained by imaging markers of tumour resistance. I have previously reviewed and summarized strategies for molecular imaging of resistance to targeted anti-cancer therapies (301). Several groups have developed imaging agents to detect P-glycoprotein (Pgp) overexpression, the protein product of the multidrug resistance gene 1 (MDR1). Pgp is a 170 kDa plasma membrane protein involved in the outward transport of lipophilic cations as well as numerous cytotoxic anti-cancer drugs (168). Overexpression of Pgp occurs in several tumour types, leading to the development of multidrug resistance. Current methods to measure Pgp at the protein or mRNA level do not always correlate with the activity of Pgp, as functionality is mediated by mutations, as well as the phosphorylation status of the protein (302). Imaging with radiopharmaceuticals that are transported by MDR1 PgP may thus provide a means to noninvasively detect functional Pgp expression in vivo. As previously discussed (section 1.7.2), several myocardial imaging agents such as $^{99m}$Tc-sestamibi, $^{99m}$Tc-tetrofosmin, and $^{99m}$Tc-furifosmin are Pgp transport substrates and are thus being investigated as Pgp imaging agents. The initial uptake of these agents in tumours is nonspecific; however, the efflux kinetics are reflective of the level of functional Pgp. Ciarmiello et al successfully imaged functional Pgp in BC patients using $^{99m}$Tc-sestamibi, and demonstrated that a rapid tumour clearance of $^{99m}$Tc-sestamibi was correlated with a lack of response to neoadjuvant chemotherapy (303). The PET analogue $^{94m}$Tc-sestamibi has been studied preclinically for imaging Pgp activity in KB 3-1 (Pgp-) and KB 8–5 (Pgp +) tumour-bearing nude mice (304). Other PET agents for probing Pgp are also being investigated, such as $^{11}$C-colchicine, $^{11}$C-verapamil, and $^{11}$C-daunomycin (302).

Resistance to trastuzumab has been associated with upregulation of the insulin-like growth factor receptor (IGF-1R). Some clinical studies have found an association between IGF-1R expression and a lack of response to trastuzumab (114). The ability to non-invasively detect IGF-1R overexpression in patients may provide a tool to predict patients with de novo or acquired resistance to trastuzumab. Our group has shown that the tumour uptake of $^{111}$In-IGF-1(E3R), an analogue which does not bind insulin growth factor-1 (IGF-1) binding proteins (e.g. IGFBP-3) correlates with the level of IGF-1R expression on human BC xenografts in athymic
mice and could be imaged using SPECT (305). Imaging molecular mechanisms of resistance may be useful for predicting resistance or monitoring response to molecularly targeted therapies.

1.10 Hypotheses

Molecular imaging techniques may be useful for non-invasive assessment of HER2 overexpression as well as monitoring the response and resistance of HER2-positive BC to treatment with trastuzumab and may have the potential for early evaluation of treatment efficacy. These procedures could provide a means to more accurately select patients for treatment with this drug. Preclinical studies of various HER2 imaging agents have demonstrated specific tumour uptake in HER2-positive tumours and minimal uptake in HER2-negative or low-expressing tumour models, however few studies have quantitatively correlated uptake of HER2 imaging agents with various levels of HER2 expression to determine the relationship between tumour uptake and HER2 expression levels (receptors/cell) and how these correlate with clinically relevant measures of HER2 expression (e.g. IHC, FISH). Furthermore, no studies have evaluated how different levels of tumour uptake correlate with response of those tumours to treatment with trastuzumab. Further research is required to define the relationship between HER2 expression levels, uptake of HER2 imaging agents and response to treatment.

The hypotheses of this thesis were:

1) Molecular imaging using $^{111}$In-labeled anti-HER2 antibodies may be useful in non-invasively assessing the HER2 expression level of BC as well as changes in HER2 expression following therapy with trastuzumab and may be useful in predicting or monitoring response of BC to treatment with trastuzumab.

2) Treatment of HER2-positive human BC with trastuzumab may result in changes in tumour glucose utilization that could be monitored non-invasively by PET using $^{18}$F-FDG as an early indication of therapeutic response.
1.11 Specific Aims

To test these hypotheses, the specific aims were:

1) To generate $^{111}$In-labeled trastuzumab ($^{111}$In-DTPA-trastuzumab) and to evaluate its purity by bioanalytical methods and its receptor binding affinity by radioligand binding assays in breast cancer cell lines that overexpress HER2.

2) To investigate the relationship between HER2 receptor expression levels, tumour uptake of $^{111}$In-DTPA-trastuzumab IgG and tumour response to trastuzumab

3) To determine if trastuzumab-mediated HER2 receptor downregulation and therapeutic response could be detected by molecular imaging using $^{111}$In-labeled pertuzumab ($^{111}$In-DTPA-pertuzumab).

4) To evaluate whether a therapeutic response to trastuzumab in human breast cancer xenografts in athymic mice results in changes in tumour glucose utilization that could be detected by PET with $^{18}$F-FDG.

1.12 Overviews of chapters 2, 3, 4 and 5

The studies addressing the above specific aims are described in Chapters 2-4 of the thesis. Chapter 2 describes the synthesis and in vitro characterization of $^{111}$In-DTPA-trastuzumab as well as the relationship between HER2 expression level and tumour uptake of the radiopharmaceutical in a panel of well-characterized s.c. human BC xenografts in athymic mice with increasing levels of HER2. This chapter also demonstrates the relationship between tumour uptake of $^{111}$In-DTPA-trastuzumab and response to trastuzumab in human BC xenografts with increasing HER2 expression. Chapter 3 demonstrates that one of the proposed mechanisms of action of trastuzumab, receptor downregulation, can be sensitively detected by molecular imaging using $^{111}$In-DTPA-pertuzumab, a second anti-HER2 antibody which binds to a different epitope of HER2 than trastuzumab and therefore is useful for measuring HER2 density in the
presence of the drug. In this chapter, changes in HER2 density following treatment with trastuzumab were measured *in vitro* by saturation receptor binding assays using $^{111}$In-DTPA-pertuzumab, as well as confocal microscopy and flow cytometry using a fluorescently labeled anti-HER2 antibody (clone 24.7). Changes in HER2 density were also evaluated *in vivo* in athymic mice bearing HER2-positive BC xenografts by microSPECT/CT imaging using $^{111}$In-DTPA-pertuzumab following treatment with trastuzumab. In Chapter 4, PET imaging using $^{18}$F-FDG was investigated as a tool to predict response to trastuzumab based on changes in tumour glucose utilization of HER2-positive and negative human BC xenografts in athymic mice. In Chapter 5, the overall conclusions are presented and discussed and future research is proposed.
CHAPTER TWO

Associations Between the Uptake of $^{111}$In-DTPA-Trastuzumab, HER2 Density and Response to Trastuzumab (Herceptin) in Athymic Mice Bearing Subcutaneous Human Tumour Xenografts

All experiments and analyses of data were carried out by Kristin McLarty except for the FISH (Chun K) and the IHC (Done SJ). The gamma camera imaging was performed with the assistance of Scollard D.
2.0 Abstract

**Purpose:** To investigate the associations between uptake of $^{111}$In-DTPA-trastuzumab, tumour HER2 density and response to trastuzumab (Herceptin) of human breast cancer (BC) xenografts in athymic mice.

**Methods:** The tumour uptake of $^{111}$In-DTPA-trastuzumab in athymic mice bearing BC xenografts with increasing HER2 density (0 to 3+) was evaluated. Specific uptake ratios were established in biodistribution (SUR) and imaging studies (ROI-SUR) using $^{111}$In-labeled mouse IgG ($^{111}$In-DTPA-mIgG). Further corrections were made for circulating radioactivity using tumour-to-blood ratios defined as a localization index [LI] and region-of-interest localization index [ROI-LI], respectively. Mice were treated with trastuzumab (Herceptin). A tumour growth inhibition index (TGI) was calculated and relative TGIs calculated by dividing the TGI of control by that of trastuzumab-treated mice.

**Results:** Strong, non-linear associations with HER2 density were obtained if the uptake of $^{111}$In-DTPA-trastuzumab was corrected for non-specific IgG localization (i.e. SUR; $r^2 = 0.99$) and circulating radioactivity (i.e. LI; $r^2 = 0.87$), but without these corrections, the association between HER2 density and tumour uptake was poor ($r^2 = 0.22$). There was a strong association between ROI-SUR and ROI-LI values and HER2 expression ($r^2 = 0.90$ and $r^2 = 0.95$, respectively. All tumours were imaged. Relative TGI values were associated with increasing uncorrected tumour uptake of $^{111}$In-DTPA-trastuzumab but not always with HER2 density (i.e. MCF-HER2-18 cells with trastuzumab-resistance).

**Conclusion:** HER2 expression (0 to 3+) can be differentiated using $^{111}$In-DTPA-trastuzumab, but requires correction of tumour uptake for non-specific IgG localization and circulating radioactivity. The uncorrected uptake of $^{111}$In-DTPA-trastuzumab was associated with tumour response to trastuzumab.
2.1 Introduction
Trastuzumab (Herceptin®) is a humanized IgG1 monoclonal antibody (mAb) which exploits the overexpression of the HER2 transmembrane receptor tyrosine kinase found in about 20% of cases of breast cancer (BC). This immunotherapeutic agent has clinical benefit in patients with metastatic HER2-positive disease both as a single agent and in combination with chemotherapy (66, 68). Trastuzumab has also recently been approved as an adjuvant treatment for early-stage BC based on improved disease-free and overall survival as well as decreased risk of recurrence (27). Patients are selected for trastuzumab-based therapies by assessment of tumour HER2 overexpression by immunohistochemical (IHC) staining or probing HER2 gene amplification using fluorescence in situ hybridization (FISH) (44). Despite pre-selection of patients with HER2-positive tumours, only a minority respond to the drug as a single agent (12-35%) (120). Response rates improve to 40-60% when trastuzumab is combined with anthracyclines or taxanes (68, 69).

Inherent in the restriction of trastuzumab to patients with tumours that overexpress HER2 is the assumption that tumour uptake of the drug is proportional to HER2 density, and that uptake is directly correlated with therapeutic response. Indeed, Phase II clinical trials of trastuzumab revealed that the overall response rate to trastuzumab was higher in those with HER2 highly-overexpressing tumours (IHC score 3+) compared to those with moderate HER2 density (IHC score 2+), presumably due to higher tumour uptake (68, 69). Molecular imaging using radiolabeled forms of trastuzumab could potentially probe the level of tumour HER2 expression as well as directly visualize the delivery of the drug to tumours (at least administered in a single radiotracer dose) and thus may be useful for predicting patient response to trastuzumab (301). This premise was previously examined by Behr et al. in a study of 20 patients with metastatic BC, who found that 11 patients with tumour uptake of $^{111}$In-DTPA-trastuzumab responded to trastuzumab, whereas only one patient without tumour uptake benefited (222). Many forms of trastuzumab (i.e. IgG, Fab, and scFv fragments), as well as anti-HER2 affibodies, minibodies and diabodies, labeled with single γ-photon-emitters (e.g. $^{111}$In, $^{99m}$Tc, $^{131}$I) or positron-emitters (e.g. $^{68}$Ga, $^{124}$I and $^{89}$Zr) have been investigated for imaging HER2-positive tumours both preclinically (45, 224, 227, 228, 234, 239, 249, 252, 258) and clinically (222-224). However, a limitation of these studies was that they did not attempt to define the quantitative associations between radiopharmaceutical uptake and tumour HER2 density, especially for tumours with a wide range of receptor expression (i.e. IHC scores 0 to
3+), nor did they examine the relationship between these parameters and tumour response to trastuzumab. Our objective was therefore to conduct the first preclinical study, to our knowledge, investigating these important associations for $^{111}$In-DTPA-trastuzumab, in a panel of human BC xenografts in athymic mice with widely varying HER2 expression. HER2 density was characterized by radioligand binding assays as well as by more clinically relevant tests (i.e. IHC and FISH) to allow interpretation of the results in the clinical context of patient tumour characteristics. $^{111}$In-DTPA-trastuzumab IgG was used for these studies because it was believed that it would be the most representative of trastuzumab and therefore, the most suitable for establishing these associations.

2.2 Materials and Methods

2.2.1 Breast cancer (BC) cells

Human BC cells (MDA-MB-231, MDA-MB-361, SKBr-3, and BT-20) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). A variant of BT-474 cells with stable bimodal (assessed by repeated HER2 characterization after multiple cell passages) and overall low (1+) HER2 expression was used. To distinguish this BT-474 variant from those reported that have higher HER2 density (239), these cells were annotated as BT-474$_{HET}$. Stably transfected MCF/HER2-18 cells were donated by Dr. Mien-Chie Hung (MD Anderson Cancer Center, Houston, TX) (306). Cells were cultured in the recommended media supplemented with 10-20% fetal bovine serum (FBS, Sigma-Aldrich). MCF/HER2-18 cells were supplemented with G418 sulphate (0.5 mg/mL; Geneticin®, Invitrogen, Carlsbad, CA).

2.2.2 Preparation and characterization of $^{111}$In-DTPA-trastuzumab

Trastuzumab (Herceptin®, Hoffmann-La Roche, Mississauga, ON) or non-specific mouse IgG (mIgG; Sigma-Aldrich Product I5381) was derivatized with a 4-fold excess of diethylenetriaminepentaacetic acid (DTPA) dianhydride (Sigma-Aldrich) (307). The conjugation efficiency and DTPA substitution level were calculated as described previously (239). DTPA-trastuzumab was purified and reconcentrated to 10 mg/mL in 1 mol/L CH$_3$COONa buffer by ultrafiltration on a Microcon YM-30 device (Amicon, Billerica, MA). DTPA-trastuzumab was labeled with $^{111}$InCl$_3$ (MDS Nordion, Kanata, ON), to a specific activity of 0.02-1 MBq/µg. $^{111}$In-DTPA-trastuzumab was purified on a Sephadex G-50 mini-column.
The final radiochemical purity was >97% measured by instant thin layer-silica gel chromatography (ITLC-SG) (239) and size-exclusion high performance liquid chromatography (SE-HPLC) performed on a BioSep SEC-S2000 column (300 × 7.8 mm; Phenomenex, Torrance, CA) eluted with 100 mM NaH₂PO₄ buffer, pH 7.0 at a flow rate of 0.8 mL/min using a Series 200 pump (PerkinElmer, Wellesley, MA) interfaced with a diode array detector (PerkinElmer) set at 280 nm and a Radiomatic 610TR flow scintillation analyzer (PerkinElmer).

The immunoreactive fraction (IRF) of ¹¹¹In-DTPA-trastuzumab was measured by the Lindmo method (308). Briefly, 10 ng of ¹¹¹In-DTPA-trastuzumab was incubated with 9.3 × 10⁵ to 3.0 × 10⁷ SKBr-3 cells/mL in 500 µl of serum-free cell culture media for 3 h at room temperature. Cells were rinsed three times to remove unbound radioactivity. The cell pellets containing bound ¹¹¹In-DTPA-trastuzumab were counted in a γ-counter (Wizard 3, PerkinElmer). Non-specific binding was estimated in the presence of 100-fold excess of unlabeled trastuzumab. Bound/total radioactivity was plotted vs. the inverse of cell concentration. The IRF was obtained from the inverse of the intercept on the ordinate as reported by Lindmo (308).

2.2.3 Saturation receptor-binding assays

The HER2 receptor density (average number of receptors/cell) on BC cells and the dissociation constants (Kₐ) for ¹¹¹In-DTPA-trastuzumab were measured in radioligand saturation receptor-binding assays. Approximately 5 × 10⁴ HER2 overexpressing SKBr-3 or MCF/HER2-18 cells were plated overnight in 24-well plates (Sarstedt, Montreal, QC). The adherent cells were then incubated in serum-free culture media with increasing concentrations of ¹¹¹In-DTPA-trastuzumab (0-150 nmol/L, 0.02 MBq/µg) in a total volume of 500 µL for 3 h at 4°C. Unbound radioactivity was removed and the cells rinsed twice with ice-cold PBS, pH 7.0. The cells were dissolved in 0.1 mol/L NaOH and the cell-bound radioactivity counted in a γ-counter. Kₐ values and the concentration of ¹¹¹In-DTPA-trastuzumab required to saturate the receptors (Bₘₐₓ) were calculated by fitting a plot of cell-bound ¹¹¹In-DTPA-trastuzumab (nmol) vs. the concentration of unbound radioligand (nmol/L, corrected for IRF) to a 1-site saturation binding model using Prism® Ver. 4.0 software (GraphPad Software, San Diego, CA). This model fitting corrects the total binding for non-specific binding by assuming that it is proportional to radioligand concentration. Bₘₐₓ values (nmol) were converted to the number of HER2 receptors/cell. The HER2 expression of MDA-MB-231, BT-20, MDA-MB-361 and BT-474₇ cells were
measured in 1-point binding assays performed by incubating $4 \times 10^5$ cells/well in 6-well plates with 2 mL of 60 nmol/L of $^{111}$In-DTPA-trastuzumab sufficient to saturate the receptors. These assays were conducted in the presence (non-specific binding) or absence (total binding) of a 100-fold molar excess of unlabeled trastuzumab. $B_{\text{max}}$ was similarly calculated and converted to the number of receptors/cell.

2.2.4 Immunohistochemistry (IHC)

IHC staining for HER2 was performed in triplicate on human BC cell lines. Cell pellets were coated in 3% agar and the agar blocks were fixed in 10% neutral buffered formalin for 24 h before being paraffin-embedded. Sections (4 µm) were dewaxed and rehydrated prior to antigen retrieval. Endogenous peroxidase and biotin activities were blocked using 3% H$_2$O$_2$ and an avidin/biotin blocking kit, respectively (Vector Laboratories, Burlington, ON). Sections were then blocked for 15 min with 10% normal serum and incubated with a mouse anti-c-erbB-2 monoclonal antibody (CB11; Novocastra Laboratories, Newcastle, UK; 1/100) for 1 h at room temperature (RT), followed by an anti-mouse biotinylated secondary antibody (Vector Laboratories) for 30 min and a horseradish peroxidase-conjugated ultrastreptavidin labeling reagent (ID Labs$^\text{TM}$ Inc, London, ON) for 30 min. Sections were washed twice with PBS and the reaction product was visualized using NovaRed solution (Vector Laboratories) and counterstained lightly with Mayer’s hematoxylin. Finally, sections were dehydrated, cleared in xylene, and mounted in Permount (Fisher Scientific, Ottawa, ON). Negative control sections were prepared with omission of the primary anti-c-erbB-2 antibody. All slides were scored based on the percentage of moderate-to-strongly staining cells with complete membrane staining.

2.2.5 Fluorescence in situ hybridization (FISH)

FISH was performed on BC cells using the PathVysion $HER$-2 probe (Abbott Molecular, Des Plaines, IL) according to the manufacturer’s instructions. The kit consists of a $HER$-2 gene DNA probe labeled with SpectrumOrange, which maps to 17q11.2-q12, and a $CEP17$ chromosome 17 specific alpha-satellite DNA probe labeled with SpectrumGreen. Fluorescence signals were detected with the Leica DMLB fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using DAPI, FITC and Texas Red filters. Representative cells were imaged digitally
using the CytoVision (Applied Imaging, San Jose, CA) image capturing system. Sixty nuclei were scored for each cell line. A \( HER2:CEP17 \) ratio of \( \geq 2.2 \) was considered HER2 amplified (44).

2.2.6 Tumour and normal tissue uptake of \(^{111}\text{In-DTPA-trastuzumab}\)

The tumour and normal tissue uptake of \(^{111}\text{In-DTPA-trastuzumab}\) were determined in female athymic CD1\( nu/nu \) mice (Charles River, Wilmington, MA) bearing s.c. MDA-MB-361 BC xenografts. Mice were inoculated s.c. in the right thigh with \( 1 \times 10^7 \) cells in 100 \( \mu\)L of growth medium mixed with 100 \( \mu\)L of Matrigel (BD Biosciences) at 24 h after s.c. implantation of a 0.72 mg, 60-day sustained-release 17\( \beta \)-estradiol pellet (Innovative Research of America, Sarasota, FL). Once the tumours had reached an average volume of 240 mm\(^3\), groups of 4 mice received an i.v. (tail vein) injection of \(^{111}\text{In-DTPA-trastuzumab}\) or \(^{111}\text{In-DTPA-mIgG}\) (10 \( \mu\)g, 0.2-0.4 MBq) in 200 \( \mu\)L of Sodium Chloride Injection USP. One group of mice received an 80-fold excess of unlabeled trastuzumab 24 h prior to injection of \(^{111}\text{In-DTPA-trastuzumab}\) to assess the specificity of tumour uptake. At 72 h post-injection (p.i.), the mice were sacrificed and the tumour, blood and normal tissues were collected, weighed and their radioactivity counted in a \( \gamma \)-counter. This time point has been shown to produce optimal tumour-to-background ratios for \(^{111}\text{In-DTPA-trastuzumab IgG}\) (227, 239). Tumour and normal tissue uptake were expressed as mean \( \pm \) SD percent injected dose/g (%ID/g).

2.2.7 Associations between tumour uptake and HER2 density

In order to evaluate the association between HER2 density (average number of receptors/cell) and the tumour uptake of \(^{111}\text{In-DTPA-trastuzumab}\) (or \(^{111}\text{In-DTPA-mIgG}\)), groups of 3-4 athymic mice were inoculated s.c. with \( 1 \times 10^7 \) MDA-MB-231, BT-20, MDA-MB-361, BT-474\(_{\text{HET}}\), or MCF/HER2-18 BC cells to establish tumour xenografts with varying HER2 expression (0 to 3+). Mice implanted with MDA-MB-361, BT-474\(_{\text{HET}}\) and MCF/HER2-18 tumour xenografts were pre-inoculated with a sustained-release 17\( \beta \)-estradiol pellet. Tumour and blood concentrations of radioactivity at 72 h p.i. were measured by \( \gamma \)-counting as described earlier. Tumour uptake was expressed as %ID/g or by a specific uptake ratio (SUR), that was defined as the uptake (%ID/g) of \(^{111}\text{In-DTPA-trastuzumab}\) divided by that of \(^{111}\text{In-DTPA-mIgG}\). In addition, blood concentrations of radioactivity were used to establish a localization index (LI), which was defined as the tumour/blood (T/B) ratio of \(^{111}\text{In-DTPA-trastuzumab}\) divided by that of \(^{111}\text{In-DTPA-mIgG}\).
2.2.8 Tumour imaging with $^{111}$In-DTPA-trastuzumab

Imaging was performed at 72 h p.i. of 10 µg (7-10 MBq) of $^{111}$In-DTPA-trastuzumab or $^{111}$In-DTPA-mIgG in tumour-bearing athymic mice using a small field-of-view clinical γ-camera (ADAC Model TransCam, ADAC Laboratories, Milpitas, CA) fitted with a 4-mm pinhole collimator. Planar images were collected into a $256 \times 256 \times 16$ acquisition matrix for a total of 250,000 counts. Region of interest (ROI) analysis was performed and counts in tumour ROIs reported as the mean ± SD percent injected dose per pixel (%ID/pixel). Specific tumour uptake was defined as an ROI uptake ratio (ROI-SUR) calculated by dividing the mean tumour counts (%ID/pixel) for $^{111}$In-DTPA-trastuzumab by that of $^{111}$In-DTPA-mIgG. To account for blood pool radioactivity, an ROI localization index (ROI-LI) was defined as the mean ratio of tumour/heart (mediastinal radioactivity) counts for $^{111}$In-DTPA-trastuzumab divided by that of $^{111}$In-DTPA-mIgG. The principles of Laboratory Animal Care (NIH Publication No. 86–23, revised 1985) were followed and all animal studies were conducted under a protocol approved by the Animal Care Committee at the University Health Network (No. 989.2) in accordance with Canadian Council on Animal Care (CCAC) guidelines.

2.2.9 Tumour response to trastuzumab

Groups of 6-8 athymic mice bearing BC xenografts (110 ± 70 mm$^3$) were randomly assigned to treatment with trastuzumab or PBS (control group). A second group of control mice bearing MDA-MB-361 tumours were treated with non-specific human IgG (hIgG; Sigma-Aldrich Product I4506). Mice were administered trastuzumab intraperitoneally (i.p.) with a loading dose of 4 mg/kg followed by weekly doses of 2 mg/kg for 4 weeks, diluted in PBS to a final volume of 100 µL. We previously determined that trastuzumab is rapidly absorbed from an i.p. injection in mice within 24 h (unpublished data). Tumours were measured weekly using calipers and tumour volume calculated using the formula: $\text{length} \times \text{width}^2 \times 0.5$. Tumour growth was normalized to the initial tumour volume (tumour growth index; TGI) and the mean TGI was compared for trastuzumab treated vs. control mice and between groups of mice implanted with the different BC xenografts. The TGI of PBS controls was divided by the TGI of trastuzumab-treated xenografts (relative TGI) for comparisons with HER2 density or uptake of $^{111}$In-DTPA-trastuzumab.
2.2.10 Statistical analysis

Tumour uptake of $^{111}$In-DTPA-trastuzumab or $^{111}$In-DTPA-mIgG was compared between xenograft models using one-way parametric ANOVA using the Bonferroni correction for multiple comparisons (P<0.05). Scatterplots were generated for comparisons between tumour uptake, SUR, ROI-SUR, LI, and ROI-LI and HER2 density. These were fitted to a 1-site non-linear association model using GraphPad Prism 4.0 and $r^2$ values calculated. Correlations between biodistribution (SUR and LI) and imaging (ROI-SUR and ROI-LI) results were made using Pearson’s correlation (P<0.05). All other statistical comparisons were made using Student’s t-test (P<0.05).

2.3 Results

2.3.1 Preparation and characterization of $^{111}$In-DTPA-trastuzumab

Under the conditions used, trastuzumab was derivatized with 1.4 ± 0.3 DTPA groups per IgG molecule (n = 10). DTPA-derivatized trastuzumab was labeled with $^{111}$InCl$_3$ to a final radiochemical purity of >97%. SE-HPLC analysis showed one major peak ($t_R = 7.8$ min) corresponding to monomeric $^{111}$In-DTPA-trastuzumab IgG with minimal (<10%) evidence of intermolecular cross-linking ($t_R = 7.1$ min; results not shown). The IRF of $^{111}$In-DTPA-trastuzumab at infinite antigen (HER2) excess was 0.64 ± 0.04.

2.3.2 Saturation receptor-binding assays

$^{111}$In-DTPA-trastuzumab demonstrated saturable binding to HER2-overexpressing SKBr-3 and MCF/HER2-18 cells which was displaceable by a 100-fold excess of unlabeled trastuzumab (results not shown). The $K_d$ was $1.7 \pm 0.8 \times 10^{-9}$ mol/L and $1.2 \pm 0.5 \times 10^{-9}$ mol/L for SKBr-3 and MCF/HER2-18 cells, respectively (not significantly different; P>0.05). $B_{max}$ values expressed as the number of HER2 receptors/cell and the correlation with IHC and FISH results are summarized in Table 2.1. HER2 density ranged from $5.4 \pm 0.7 \times 10^4$ receptors/cell for MDA-MB-231 cells to $1.3 \pm 0.3 \times 10^6$ receptors/cell for SKBr-3 cells. The overall HER2 density of BT-474$_{HET}$ cells was $1.3 \pm 0.7 \times 10^5$ receptors/cell, although IHC and FISH confirmed that there was a bimodal distribution. MCF/HER2-18 cells displayed $1.2 \pm 0.1 \times 10^6$ HER2 receptors/cell and were used to establish tumour xenografts that exhibit high HER2 expression (3+), since SKBr-3 cells are not tumourigenic. Based on the number of receptors per cell, BC cells were assigned relative HER2 expression scores of 0 to 3+ (Table 2.1) to relate these to
more commonly used IHC staining scores that were previously assigned to cells with known HER2 densities (0: 20,000; 1+: 100,000; 2+: 500,000 and 3+: 2,000,000) (309-311).

2.3.3 Immunohistochemistry (IHC)

Greater than 10% tumour cells with moderate-to-strong, complete membrane staining by IHC (Fig. 2.1 A and Table 2.1) was considered clinically HER2-positive (312). MDA-MB-231 and BT-20 cell lines had <1% and 0%, respectively, of immunopositive cells. MDA-MB-361 cells were 45% positive and MCF/HER2-18 cells were 38% positive. BT-474HET cells showed 5% staining, while SKBr-3 cells had 98% of HER2-positive cells. Using the applied criteria, IHC classified MDA-MB-231, BT-20 and BT-474HET cells as HER2-negative and MDA-MB-361, MCF/HER2-18 and SKBr-3 cells as HER2-positive.

2.3.4 Fluorescence in situ hybridization (FISH)

FISH (Fig. 2.1 B and Table 2.1) revealed that MDA-MB-231 and BT-20 cells were not HER2 gene-amplified (HER2:CEP17 ratios <2.2), whereas MDA-MB-361 and SKBr-3 cells exhibited gene amplification ratios of >5 and >3, respectively. FISH confirmed that BT-474HET cells harbored HER2 gene-amplified (ratio of >3) and non gene-amplified (ratio of 1) cells. Despite high surface HER2 expression, MCF/HER2-18 cells did not exhibit gene amplification detected by FISH (ratio of 0.7). This may be explained by poor hybridization of the PathVysion HER-2 probe to the transfected HER2 cDNA, which is only 4.6 kb (comprising only the coding region of HER2) in contrast to its more efficient hybridization to the HER2 amplicon that is greater than 280 kb (313).
TABLE 2.1
Characterization of the HER2 density of human breast cancer cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HER2 density (receptors/cell (\times 10^5))(^a)</th>
<th>IHC (% immunopositive cells)(^b)</th>
<th>FISH (gene amplification ratio)(^c)</th>
<th>Relative HER2 expression(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>0.54 ± 0.07</td>
<td>&lt;1</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>BT-474(_{HET})</td>
<td>1.3 ± 0.7</td>
<td>5</td>
<td>&gt;3.0 (22/60)</td>
<td>1+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0 (38/60)</td>
<td></td>
</tr>
<tr>
<td>BT-20</td>
<td>1.6 ± 0.4</td>
<td>0</td>
<td>1.0</td>
<td>1+</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>5.1 ± 1.7</td>
<td>45</td>
<td>&gt;5.0</td>
<td>2+</td>
</tr>
<tr>
<td>MCF/HER2-18</td>
<td>11.6 ± 1.3</td>
<td>38</td>
<td>0.7 (51/60)</td>
<td>3+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0 (9/60)</td>
<td></td>
</tr>
<tr>
<td>SK-Br-3</td>
<td>13.4 ± 3.4</td>
<td>98</td>
<td>&gt;3.0</td>
<td>3+</td>
</tr>
</tbody>
</table>

\(^a\) HER2 density (\(B_{max}\)) was measured in a direct radioligand binding assay with \(^{111}\)In-DTPA-trastuzumab.

\(^b\) Immunohistochemistry (IHC) was scored as the percentage of cells with moderate-to-strong and complete membrane staining (>10% is considered HER2-positive).

\(^c\) Fluorescence in situ hybridization (FISH) was reported as the ratio of \(HER2\) gene copy to \(centromere\)-17 gene copy (>2.2 was considered gene-amplified). In some cell lines, the proportion of cells with these gene amplification ratios is shown in parentheses.
A

I. <1%

II. 5%

III. 0%

IV. 45%

V. 38%

VI. 98%

B

1.0

>3.0 (22/60)

1.0 (38/60)

1.0

>5.0

0.7 (51/60)

1.0 (9/60)

>3.0
Fig. 2.1. Characterization of HER2 expression in a panel of human breast cancer cells lines. (A) Immunohistochemical (IHC) staining of the cells [I. MDA-MB-231, II. BT-474HET, III. BT-20, IV. MDA-MB-361, V. MCF/HER2-18 and VI. SKBr-3; percentages of HER2-positive cells are shown]. (B) Corresponding fluorescence in situ hybridization (FISH) results [HER2:CEP17 ratios are indicated].
2.3.5 Tumour and normal tissue uptake of $^{111}$In-DTPA-trastuzumab

The tumour and normal tissue uptake of $^{111}$In-DTPA-trastuzumab at 72 h p.i. in athymic mice bearing HER2 2+ MDA-MB-361 BC xenografts is shown in Table 2.2. Tumour accumulation of radioactivity for mice receiving $^{111}$In-DTPA-trastuzumab was more than 4-fold higher than for those injected with $^{111}$In-DTPA-mIgG (23.4 ± 1.6 vs. 5.5 ± 0.6 %ID/g; P<0.001). Moreover, pre-injection of an 80-fold excess of unlabeled trastuzumab (800 µg) 24 h prior to $^{111}$In-DTPA-trastuzumab decreased tumour uptake 3.3-fold (7.0 ± 2.9 vs. 23.4 ± 1.6 %ID/g, respectively; P<0.001; Table 2.2). Tumour uptake of $^{111}$In-DTPA-trastuzumab following blocking with unlabeled trastuzumab was not significantly different than that of $^{111}$In-DTPA-mIgG (5.5 ± 0.6 %ID/g; P>0.05). The highest normal tissue concentrations of radioactivity for $^{111}$In-DTPA-trastuzumab as well as $^{111}$In-DTPA-mIgG were found in the blood, spleen, liver and kidneys (Table 2.2). Uptake in these tissues was comparable with previous reports for $^{111}$In-DTPA-trastuzumab IgG (227, 228).
TABLE 2.2
Tumour and normal tissue uptake of $^{111}$In-DTPA-trastuzumab and $^{111}$In-DTPA-mIgG at 72 hours post-injection in athymic mice bearing subcutaneous MDA-MB-361 human breast cancer xenografts. $^a$

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percent injected dose/gram (mean ± SD)</th>
<th>$^{111}$In-DTPA-trastuzumab</th>
<th>$^{111}$In-DTPA-mIgG</th>
<th>$^{111}$In-DTPA-trastuzumab (blocked) $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3.5±1.0</td>
<td>2.2±0.6</td>
<td>4.1±1.1</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>1.3±0.3</td>
<td>1.5±0.3</td>
<td>1.4±0.2</td>
<td></td>
</tr>
<tr>
<td>Lungs</td>
<td>2.2±0.6</td>
<td>2.3±0.2</td>
<td>2.4±0.3</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>4.2±0.3</td>
<td>5.9±0.5</td>
<td>4.6±1.3</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>3.2±0.8</td>
<td>6.4±1.5</td>
<td>3.1±0.9</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>0.5±0.1</td>
<td>0.8±0.1</td>
<td>0.9±0.4</td>
<td></td>
</tr>
<tr>
<td>Kidneys</td>
<td>5.7±0.8</td>
<td>9.6±0.8</td>
<td>6.1±1.2</td>
<td></td>
</tr>
<tr>
<td>Small intestines</td>
<td>1.2±0.1</td>
<td>2.1±0.3</td>
<td>1.3±0.2</td>
<td></td>
</tr>
<tr>
<td>Large intestines</td>
<td>0.8±0.1</td>
<td>1.2±0.1</td>
<td>0.9±0.1</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>0.8±0.2</td>
<td>0.9±0.3</td>
<td>0.7±0.1</td>
<td></td>
</tr>
<tr>
<td>Tumour</td>
<td>23.4±1.6</td>
<td>5.5±0.6 $^c$</td>
<td>7.0±2.9 $^c$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Groups of 3-4 mice were injected i.v. (tail vein) with 10 µg (0.2-0.4 MBq) of $^{111}$In-DTPA-trastuzumab or $^{111}$In-DTPA-mIgG.

$^b$ HER2 receptor blocking was achieved by pre-administration of 800 µg of unlabeled trastuzumab 24 h prior to injection of $^{111}$In-DTPA-trastuzumab

$^c$ P-value for comparison with the tumour uptake of $^{111}$In-DTPA-trastuzumab is <0.005 for $^{111}$In-DTPA-mIgG and <0.001 for $^{111}$In-DTPA-trastuzumab (blocked)
2.3.6 Associations between tumour uptake and HER2 density

The relationships between tumour uptake of radioactivity for $^{111}$In-DTPA-trastuzumab (uncorrected or expressed as SUR or LI) and HER2 density (average number of receptors/cell; Table 2.1) are shown in Fig. 2.2. The association between the uncorrected radioactivity and HER2 expression level was poor ($r^2=0.22$; Fig. 2.2 A). The highest tumour uptake ($23.4 \pm 1.6 \%$ID/g) was observed for MDA-MB-361 xenografts which displayed 2+ HER2 expression. Lower accumulation of $^{111}$In-DTPA-trastuzumab was found in MDA-MB-231, BT-20 and BT-474$_{HET}$ tumours ($11.4 \pm 1.4$, $9.0 \pm 1.7$ % and $13.7 \pm 1.5$ %ID/g, respectively) with fewer HER2 receptors (0 or 1+). However, the uptake of $^{111}$In-DTPA-trastuzumab in highly HER2-positive (3+) MCF/HER2-18 tumours ($13.6 \pm 3.0$ %ID/g) was similar to that in MDA-MB-231, BT-20 or BT-474$_{HET}$ xenografts. In addition, there were significant differences (ANOVA; $P<0.0001$) in the uptake of $^{111}$In-DTPA-mIgG between the various BC xenografts (Table 2.3), but no associations were found between these differences and HER2 expression (Fig. 2.2 A).

In contrast to the poor relationship found between the uncorrected tumour uptake of $^{111}$In-DTPA-trastuzumab and HER2 density (Fig. 2.2 A), there was a strong, non-linear association of the SUR values with HER2 expression ($r^2=0.99$; Fig. 2.2 B). SUR values incorporated differences in non-specific IgG uptake estimated using $^{111}$In-DTPA-mIgG, but it was noted that there were also significant differences (ANOVA; $P<0.005$) in the blood concentrations of radioactivity between groups of mice injected with $^{111}$In-DTPA-trastuzumab or $^{111}$In-DTPA-mIgG (Table 2.3). These differences in the level of circulating radioactivity may affect tumour uptake of the radiopharmaceuticals; therefore, a second LI was established. The association between LI and HER2 density was slightly weaker than the relationship between SUR and HER2 expression ($r^2=0.87$; Fig. 2.2 C).
**Fig. 2.2.** Associations between HER2 expression levels in a panel of human breast cancer xenografts and tumour uptake of $^{111}$In-DTPA-trastuzumab or $^{111}$In-DTPA-mIgG at 72 h post-injection. (A) Tumour uptake expressed as percent injected dose/g [%ID/g; mean ± SD (n=4)], (B) Tumour uptake expressed as Specific Uptake Ratios (SUR) and (C) Tumour uptake expressed as Localization Indices (LI).
TABLE 2.3

Tumour and blood radioactivity for $^{111}$In-DTPA-trastuzumab and $^{111}$In-DTPA-mIgG at 72 hours post-injection in athymic mice bearing subcutaneous human breast cancer xenografts

<table>
<thead>
<tr>
<th>Breast cancer xenograft</th>
<th>Tumour uptake (%ID/g) $^a$</th>
<th>Blood concentration (%ID/g) $^a$</th>
<th>T/B ratio (Biodistribution studies)</th>
<th>ROI T/B ratio (Imaging studies) $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trastuzumab mlG $^c$</td>
<td>Trastuzumab mlG $^d$</td>
<td>Trastuzumab mlG $^e$</td>
<td>Trastuzumab mlG $^f$</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>11.4 ± 1.4</td>
<td>8.5 ± 2.2</td>
<td>6.6 ± 1.6</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.4 ± 0.3</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>BT-474HET</td>
<td>13.7 ± 1.5</td>
<td>5.2 ± 0.3</td>
<td>4.0 ± 1.4</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.7 ± 0.6</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>BT-20</td>
<td>9.0 ± 1.7</td>
<td>6.1 ± 0.7</td>
<td>3.8 ± 1.4</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.1 ± 0.4</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>23.4 ± 1.6</td>
<td>3.5 ± 1.0</td>
<td>2.2 ± 0.6</td>
<td>6.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.6 ± 0.6</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>MCF/HER2-18</td>
<td>13.6 ± 3.0</td>
<td>3.8 ± 1.3</td>
<td>1.6 ± 0.5</td>
<td>3.8 ± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.7 ± 0.3</td>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

$a$ Tumour and blood concentrations of radioactivity (mean ± SD) were measured in biodistribution studies by $\gamma$-scintillation counting in groups of 3-4 mice.

$^c$ Tumour uptake of $^{111}$In-DTPA-trastuzumab was significantly greater than that for $^{111}$In-DTPA-mIgG (t-test; $P<0.05$) in all tumour xenografts.

$^d$ Blood concentrations of radioactivity for $^{111}$In-DTPA-trastuzumab were significantly different between the groups of mice bearing different breast cancer xenografts (ANOVA; $P<0.005$). Blood concentrations for $^{111}$In-DTPA-trastuzumab were significantly different than those for $^{111}$In-DTPA-mIgG (t-test; $P<0.05$) for mice bearing BT-20 or MCF/HER2-18 tumours.

$^e$ Blood concentrations of radioactivity for $^{111}$In-DTPA-mIgG were significantly different between groups of mice bearing different breast cancer xenografts (ANOVA; $P<0.0005$).

$^f$ T/B ratios were significantly different between $^{111}$In-DTPA-trastuzumab and $^{111}$In-DTPA-mIgG for mice bearing MDA-MB-361 or MCF/HER2-18 tumours (t-test; $P<0.05$).

$^g$ ROI T/B ratios were significantly different between $^{111}$In-DTPA-trastuzumab and $^{111}$In-DTPA-mIgG for mice bearing MDA-MB-361 tumours (t-test; $P<0.05$).
2.3.7 Tumour imaging with $^{111}$In-DTPA-trastuzumab

Tumour as well as normal tissue uptake of radioactivity in the liver, spleen, kidneys and excreted into the bladder were observed on images of mice at 72 h p.i. of $^{111}$In-DTPA-trastuzumab or $^{111}$In-DTPA-mIgG (Fig. 2.3). In all BC xenografts (except MDA-MB-231), there was significantly higher uptake of radioactivity in mice injected with $^{111}$In-DTPA-trastuzumab than in those receiving $^{111}$In-DTPA-mIgG. ROI-SUR values ranged from 0.9 ± 0.3 for MDA-MB-231 tumours (P=0.42) to 2.8 ± 0.4 for MDA-MB-361 xenografts (P=0.001) and 2.3 ± 0.8 for MCF/HER2-18 tumours (P=0.045). There was a poor association between the uncorrected uptake of $^{111}$In-DTPA-trastuzumab (%ID/pixel) and HER2 expression level ($r^2=0.09$; Fig. 2.4 A). However, there was a strong, non-linear association between ROI-SUR values with HER2 density ($r^2=0.90$; Fig. 2.4 B). This association improved slightly using ROI-LI values ($r^2=0.95$; Fig. 2.4 C) that incorporated differences in blood pool (mediastinal) radioactivity. The accuracy in measuring the relative tumour uptake of $^{111}$In-DTPA-trastuzumab and $^{111}$In-DTPA-mIgG or blood pool radioactivity on the images was shown by the strong and significant correlation between ROI-SUR and SUR values ($r=0.92$, $P=0.03$; Fig. 2.5 A) and between ROI-LI and LI values ($r=0.97$, $P=0.006$; Fig. 2.5 B).

2.3.8 Tumour response to trastuzumab

Tumour growth was strongly inhibited by trastuzumab in mice bearing s.c. MDA-MB-361 xenografts with 2+ HER2 expression. TGIs in these trastuzumab-treated mice were significantly lower than in controls treated with PBS (0.5 ± 0.2 vs. 6.9 ± 3.1, respectively; $P<0.002$) or those administered hIgG (5.3 ± 2.7; $P<0.004$). There were no significant tumour growth-inhibitory effects of trastuzumab on MDA-MB-231 or BT-20 xenografts with lower levels of HER2 (0 and 1+, respectively). TGIs for mice with these tumours treated with trastuzumab were 1.0 ± 0.6 and 1.6 ± 0.8, respectively; these were not significantly different than those for PBS-treated controls (2.5 ± 2.0 and 1.5 ± 1.0, respectively; $P>0.05$). MCF/HER2-18 xenografts with 3+ HER2 expression were not growth-inhibited by trastuzumab compared to PBS-treated controls (1.4 ± 0.5 vs. 2.6 ± 2.5, respectively; $P>0.10$). A comparison of these trastuzumab treatment outcomes with the uncorrected tumour uptake of $^{111}$In-DTPA-trastuzumab (Table 2.3) revealed that the greatest response was observed for MDA-MB-361 tumours that accumulated the highest concentration of the radiopharmaceutical. MDA-MB-231 and BT-20 tumours with 0 and 1+
HER2 expression, as well as MCF/HER2-18 xenografts (3+) exhibited 2-3 fold lower uptake of $^{111}$In-DTPA-trastuzumab than MDA-MB-361 tumours and were unresponsive.
Fig. 2.3. Posterior whole-body planar images of representative athymic mice implanted s.c. in the right hind leg with human breast cancer xenografts (white arrow) at 72 h post-injection of $^{111}$In-DTPA-trastuzumab (top row) or $^{111}$In-DTPA-mIgG (bottom row). The relative expression of the tumours are: I. MDA-MB-231 (0), II. BT-474$_{HET}$ (1+), III. BT-20 (1+), IV. MDA-MB-361 (2+), and V. MCF/HER2-18 (3+). Also visualized on the images are the liver (large arrowhead) and kidneys (small arrowhead).
Fig. 2.4. Associations between HER2 expression levels of a panel of human breast cancer xenografts and tumour uptake of $^{111}$In-DPTA-trastuzumab or $^{111}$In-DTPA-mIgG at 72 h post-injection, determined by ROI analysis. (A) Tumour uptake expressed as percent injected dose/pixel [%ID/pixel; mean ± SD (n=3)], (B) Tumour uptake expressed as region-of-interest Specific Uptake Ratios (ROI-SUR) and (C) Tumour uptake expressed as region-of-interest Localization Indices.
Fig. 2.5. Correlations between estimation of $^{111}$In concentrations by region-of-interest analysis of images and tissue biodistribution studies. (A) ROI-SUR vs. SUR and (B) ROI-LI vs. LI.
2.4 Discussion

Molecular imaging has great potential to probe the levels of therapeutically important targets in malignancies such as HER2 (301), but the associations between tumour uptake of the radiopharmaceuticals and the target receptor density, as well as the relationship between these parameters and therapeutic outcome must be established. In this study, we showed that there was a strong, non-linear association between HER2 density (average number of receptors/cell) on BC cells forming tumour xenografts in athymic mice, and the accumulation of $^{111}$In-DTPA-trastuzumab corrected for non-specific IgG localization (using SUR and ROI-SUR values) or circulating radioactivity (using LI and ROI-LI values). This strong association was found in biodistribution studies ($r^2 = 0.87-0.99$; Fig. 2.2) as well as in region-of-interest (ROI) analysis of images ($r^2 = 0.90-0.95$; Fig. 2.4). In contrast, only a poor association was found between the uncorrected tumour uptake of $^{111}$In-DTPA-trastuzumab and the HER2 expression of BC cells in these studies ($r^2 = 0.09-0.22$). Cai et al. (216) recently reported the correlation between the uptake of $^{64}$Cu-labeled cetuximab and EGFR expression in a panel of tumour xenografts in athymic mice but did not correct for non-specific IgG localization or blood flow and obtained a poorer correlation ($r^2=0.80$ for %ID/g and $r^2=0.64$ for tumour/muscle ratios) than we obtained by applying these corrections. The uptake of $^{111}$In-DTPA-trastuzumab was specific as revealed by the 4-fold significantly lower accumulation of $^{111}$In-DTPA-mIgG in MDA-MB-361 tumours (2+ HER2 expression), and the 3-fold significantly lower uptake of $^{111}$In-DTPA-trastuzumab following HER2 receptor blocking by pre-administration of an 80-fold excess of unlabeled trastuzumab (Table 2.2).

The non-linear associations found between SUR or LI and ROI-SUR or ROI-LI and HER2 density may be due to the limited and small mass (10 µg) of $^{111}$In-DTPA-trastuzumab administered; this mass may have been insufficient to saturate all of the receptors on the 3+ MCF/HER2-18 cells. This non-linearity may also reflect quantitative differences among the tumours in receptor-independent processes that control the uptake of immunoglobulins (314). Notably, the accumulation of $^{111}$In-DTPA-trastuzumab in MCF/HER2-18 tumours was 2-fold significantly lower than in MDA-MB-361 xenografts (Table 2.3), despite their 2-fold higher HER2 density (Table 2.1). Furthermore, the uptake of non-specific $^{111}$In-DTPA-mIgG was 2-3 fold lower in MCF/HER2-18 tumours than in other BC xenografts (Table 2.3). Non-linearity in
tumour uptake of $^{111}$In-DTPA-trastuzumab could similarly occur in patients administered the low masses (5 mg) used for imaging studies (223, 315), and/or due to physiological limitations in delivery of IgGs to lesions (314). Underestimation of the level of HER2 expression in tumours may occur if a linear relationship between tumour uptake of $^{111}$In-DTPA-trastuzumab and HER2 density is assumed. In addition, differences in the proportion of non-specific IgG uptake or differences in the levels of circulating radioactivity may affect tumour uptake. Correction for these effects in patients may be difficult, but could potentially be achieved in a single study by imaging using $^{111}$In-DTPA-trastuzumab and $^{99m}$Tc-labeled human IgG (hIgG) (316, 317). Analogous approaches have been used for correction of the tumour uptake of $^{131}$I-anti-carcinoembryonic antigen (CEA) monoclonal antibodies in patients for contributions due to blood pool, using $^{99m}$Tc-human serum albumin (318, 319). Nonetheless, differences in the radiochemistry of $^{99m}$Tc- and $^{111}$In-labeled hIgG may contribute to inaccuracies in estimating non-specific IgG localization, thus requiring two separate studies with $^{111}$In-DTPA-trastuzumab and $^{111}$In-labeled hIgG which may not be feasible. Alternatively, antibody fragments (e.g. Fab or scFv) of trastuzumab which may have a lower proportion of non-specific tumour uptake than the intact IgG, could be used to evaluate HER2 expression without these corrections (239, 257). Nonetheless, the results of our study support the premise that provided that appropriate corrections are applied, four relatively broad but therapeutically important levels of HER2 expression (0, 1, 2 or 3+) could potentially be differentiated in patients by imaging with $^{111}$In-DTPA-trastuzumab.

Our study further revealed an association between the uncorrected accumulation of $^{111}$In-DTPA-trastuzumab in BC xenografts and response to trastuzumab, irrespective of the HER2 expression level. The greatest response was found in MDA-MB-361 tumours with intermediate (2+) HER2 density; these tumours had a 2-3 fold higher uptake of $^{111}$In-DTPA-trastuzumab than in the other xenografts, including MCF/HER2-18 (3+) tumours. These results agreed with the report by Behr et al. in which the uncorrected tumour uptake of $^{111}$In-DTPA-trastuzumab on SPECT images was associated with response to trastuzumab in BC patients (222). Our results examining MDA-MB-231, BT-20 and MDA-MB-361 tumours with 0, 1+ or 2+ HER2 expression, respectively, also agreed with current eligibility criteria for trastuzumab in which only patients with tumours with at least 2+ HER2 density are expected to benefit (27, 44, 68, 69, 120). The discordant therapeutic response for HER2 3+ MCF/HER2-18 tumours treated with trastuzumab may be due to low uptake of trastuzumab, perhaps predicted by their 2-fold lower accumulation of $^{111}$In-DTPA-trastuzumab (Table 2.3) and/or the presence of trastuzumab-
resistance mechanisms in these cells [i.e. insulin-like growth factor-1 receptors (IGF-1R)] (320, 321). Some clinical studies have found an association between IGF-1R expression and a lack of response to trastuzumab (322). Thus, a similar discordant result may occur in patients between response to trastuzumab, tumour uptake of $^{111}$In-DTPA-trastuzumab on images and HER2 overexpression in tumour biopsies. Additional molecular imaging probes that elaborate resistance pathways would be helpful (305). Further examination of a larger panel of tumour xenografts with 0 to 3+ HER2 expression may be required to fully understand the associations between tumour uptake of $^{111}$In-DTPA-trastuzumab, HER2 density and response to trastuzumab.

Ultimately, the use of molecular imaging to assess the level of HER2 expression (0 to 3+) in tumours or for predicting therapeutic outcome will depend on the ability to detect lesions. Clinically, SPECT has limitations in quantifying radionuclide uptake in tissues, including a low $\gamma$-photon detection efficiency as well as attenuation by overlying tissues (particularly for deep-seated lesions), contributions from scattered $\gamma$-photons and partial volume effects (323, 324). One study found that only 45% of known lesions in 15 BC patients were detected by SPECT using $^{111}$In-DTPA-trastuzumab; but occult lesions were found in 13 patients (223). In addition, the regional detection rate was higher at 73% (Dr. Pieter Jager, personal communication, November 2007), indicating that many patients had at least one lesion imaged and characterized as HER2-positive. These findings suggest that imaging with $^{111}$In-DTPA-trastuzumab may be valuable for making therapeutic decisions even if not all lesions are detected. Furthermore, several investigators have reported that $^{111}$In concentrations in tissues in patients or animals can be accurately quantified by SPECT provided that the limitations are addressed (325, 326).

Encouragingly, we found strong correlations between ROI-SUR and SUR values ($r=0.92$, $P=0.03$; **Fig. 2.5 A**) and between ROI-LI and LI values ($r=0.97$, $P=0.006$; **Fig. 2.5 B**), indicating that reliable estimates of $^{111}$In concentrations, at least in small animal tumour xenograft models are possible. Nonetheless, the sensitivity of detection of HER2-positive tumours as well as accurate quantification of tumour and normal tissue concentrations of radiolabeled trastuzumab would be substantially improved by PET. A recent study reported visualization of HER2-positive tumours using trastuzumab labeled with the positron-emitter, $^{89}$Zr (224).
2.5 Conclusion

We conclude that imaging with $^{111}$In-DTPA-trastuzumab may be valuable for assessing relative HER2 expression in lesions (i.e. 0 to 3+) in BC patients, provided that appropriate corrections are applied for non-specific IgG tumour localization as well as circulating radioactivity. The uncorrected tumour uptake of the radiopharmaceutical was associated with response to treatment with trastuzumab (Herceptin) in the limited panel of tumour xenografts examined, but it is important to recognize that compensatory trastuzumab-resistance mechanisms (e.g. IGF-1R overexpression) can cause discordance between HER2 density, tumour uptake of $^{111}$In-DTPA-trastuzumab on the images and response to the drug. Evaluation of a larger panel of tumour xenografts expressing 0 to 3+ HER2 density is warranted to fully understand these relationships.
CHAPTER THREE

MicroSPECT/CT with \(^{111}\text{In-DTPA-Pertuzumab}\) Sensitively Detects Trastuzumab-Mediated HER2 Downregulation and Tumour Response in Athymic Mice Bearing MDA-MB-361 Human Breast Cancer Xenografts

All experiments and analyses of data were carried out by Kristin McLarty except for the confocal microscopy (Cai Z) and scoring of the IHC (Done SJ).
3.0 Abstract

**Purpose:** Pertuzumab is a HER2 dimerization inhibitor which binds to an epitope unique from that of trastuzumab (Herceptin®). Our objective was to determine if single-photon emission tomography (SPECT) imaging with $^{111}$In-DTPA-pertuzumab could sensitively detect an early molecular response to trastuzumab manifested by HER2 downregulation and a later tumour response revealed by a decreased number of HER2-positive viable tumour cells.

**Methods:** Changes in HER2 density in SKBr-3 and MDA-MB-361 BC cells exposed to trastuzumab (14 μg/mL) *in vitro* were measured by saturation binding assays using $^{111}$In-DTPA-pertuzumab and by confocal immunofluorescence microscopy and flow cytometry with FITC-labeled HER2/neu antibodies. Imaging of HER2 downregulation was studied *in vivo* in athymic mice with s.c. MDA-MB-361 tumours treated for 3 d with 4 mg/kg of trastuzumab or non-specific hIgG or PBS. Imaging of tumour response to trastuzumab was studied in mice bearing s.c. MDA-MB-361 xenografts treated with 4 mg/kg trastuzumab followed by weekly doses of 2 mg/kg, non-specific hIgG or rituximab (Hoffman-La Roche) or PBS. Mice were imaged on a microSPECT/CT system at 72 h p.i. of $^{111}$In-DTPA-pertuzumab. Tumour and normal tissue biodistribution was determined.

**Results:** $^{111}$In-DTPA-pertuzumab saturation binding to SKBr-3 and MDA-MB-361 cells was significantly decreased at 72 h following exposure *in vitro* to 14 μg/mL trastuzumab compared to untreated controls (62 ± 2 %; p<0.0001 and 32 ± 9 %; p<0.0002, respectively). Following 3 days of trastuzumab *in vivo* tumour uptake of $^{111}$In-DTPA-pertuzumab decreased 2-fold in trastuzumab vs. PBS-treated mice (13.5 ± 2.6 vs. 28.5 ± 9.1 %ID/g, respectively; p<0.05). There was also a 2-fold decreased tumour uptake in trastuzumab vs. PBS-treated mice by image volume-of-interest (VOI) analysis (p=0.05), suggesting trastuzumab-mediated HER2 downregulation. Following 3 weeks of trastuzumab, tumour uptake of $^{111}$In-DTPA-pertuzumab decreased 4.5-fold compared to PBS-treated mice (7.6 ± 0.4 vs. 34.6 ± 9.9 %ID/g, respectively; p<0.001); this decrease was associated with almost completed eradication of HER2-positive tumour cells determined immunohistochemically.

**Conclusion:** $^{111}$In-DTPA-pertuzumab sensitively imaged HER2 downregulation following 3 days of treatment with trastuzumab as well as detected a reduction in viable HER2-positive tumour cells following 3 weeks of therapy in MDA-MB-361 human breast cancer xenografts.
3.1 Introduction

Molecular imaging is a powerful new tool which has great potential for aiding in the optimal use of novel targeted cancer therapies through: i) revealing the expression of target receptors in situ on lesions throughout the body, ii) probing downstream treatment-induced molecular events, thus providing early mechanistic evidence of tumour response, and iii) monitoring the prior existence or emergence of resistance pathways implicated in treatment failure (301). Trastuzumab (Herceptin®, Roche Pharmaceuticals) is a humanized IgG1 monoclonal antibody (mAb) approved for the treatment of early and advanced breast cancer (BC) which overexpresses the HER2 transmembrane tyrosine kinase (327, 328). HER2 positivity is evaluated in a primary BC biopsy by immunohistochemical (IHC) staining for HER2 protein or probing HER2 gene amplification by fluorescence in situ hybridization (FISH) (44). Despite recent revisions to the standards for HER2 testing (44), only about one in two patients with metastatic HER2-amplified tumours benefit from trastuzumab combined with paclitaxel or anthracyclines (68). Novel approaches that more sensitively and accurately predict or monitor response to trastuzumab are needed.

HER2 expression can be imaged in situ in tumours by single-photon emission tomography (SPECT) or positron emission tomography (PET) using radiolabeled mAbs, antibody fragments [e.g. Fab or F(ab’)_2], engineered antibody forms (e.g. minibodies, diabodies or scFvs) or affibodies (234, 239, 249, 252, 258, 329, 330).

Response or resistance to trastuzumab are manifested at the molecular level, yet few studies have attempted to probe these pivotal early downstream molecular events. The routes through which trastuzumab exerts its anti-tumour effects are complex and multifactorial (80). Nonetheless, one putative downstream mechanism-of-action is to promote HER2 downregulation, thereby diminishing receptor-activated mitogenic signaling (331). In this study, we investigated ^111^In-DTPA-pertuzumab as a sensitive probe of trastuzumab-mediated HER2 downregulation in human BC xenografts in athymic mice as well as their response to treatment with the drug. Our hypothesis was that trastuzumab-mediated downregulation of HER2 would result in a decreased tumour imaging signal for ^111^In-DTPA-pertuzumab, which would not be effected by the binding of trastuzumab to HER2 (i.e. receptor blocking), due to the unique epitopes of these two antibodies. We further hypothesized that the response to trastuzumab would be manifested over a longer time period by a decreased imaging signal compared to pre-treatment due to eradication of viable HER2-overexpressing tumour cells.
3.2 Materials and Methods

3.2.1 Breast cancer cells
MDA-MB-361, MDA-MB-231 and SKBr-3 cells were purchased from the American Type Culture Collection (Manassas, VA). MDA-MB-361 and MDA-MB-231 were cultured in Leibovitz L15 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20% and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), respectively, under a 100% air atmosphere at 37°C. SKBr-3 cells were cultured in RPMI 1640 medium supplemented with 10% FBS under a 5% CO\textsubscript{2} atmosphere at 37°C.

3.2.2 \textsuperscript{111}In-DTPA-pertuzumab
Pertuzumab (Omnitarg\textsuperscript{®}, Genentech Inc., South San Francisco, CA) was derivatized with a 4-fold molar excess DTPA dianhydride (Sigma-Aldrich). Briefly, a 2.7 mmols/L solution of DTPA in anhydrous DMSO was reacted with 34 \textmu L of pertuzumab (15 \textmu g/\textmu L in 50 mM NaHCO\textsubscript{3} buffer, pH 8.0) for 1 h at room temperature (RT). Excess DTPA was removed by ultrafiltration on a Microcon YM-50 device (Millipore, Billerica, MA), with an excess of 1 mol/L sodium acetate buffer, pH 6.0. DTPA substitution was measured as previously reported (7). Purified DTPA-pertuzumab was labeled with \textsuperscript{111}In by incubation with \textsuperscript{111}In-acetate for 1 h at RT. The reaction mixture was purified on a Sephadex G-50 mini-column (Sigma-Aldrich). The final radiochemical purity (RCP) was measured by instant thin layer-silica gel chromatography (ITLC-SG; Pall Life Sciences, Mississauga, ON) developed in 100 mmols/L of sodium citrate, pH 5.0 (239). The RCP was confirmed by size-exclusion HPLC (SE-HPLC) as previously reported (330). The immunoreactivity of \textsuperscript{111}In-DTPA-pertuzumab was determined by measuring its dissociation constant (K\textsubscript{d}) and receptor density (B\textsubscript{max}) in direct saturation binding assays using SKBr-3 cells (330).

3.2.3 Competition for HER2 binding on breast cancer cells
Competition for binding of \textsuperscript{111}In-DTPA-pertuzumab to HER2 in the presence of trastuzumab was studied by seeding $8 \times 10^4$ SKBr-3 or $1 \times 10^5$ MDA-MB-361 cells in 24-well plates (Sarstedt, Montreal, QC) and culturing overnight. The culture medium was removed and the adherent cells rinsed with ice cold phosphate-buffered saline, pH 7.0 (PBS). Cells were then
incubated with 500 pmols/L of $^{111}$In-DTPA-pertuzumab in the presence of increasing concentrations (0 to 0.5 μmol/L) of trastuzumab in 400 μL of serum-free medium for 3 h at 4 °C. The medium was removed, the cells rinsed and finally solubilized in 135 μL of 100 mmols/L NaOH. Solubilized cells were transferred to γ-counting tubes and the wells rinsed 2 × with PBS and these rinses combined with those previously removed. The cell-bound radioactivity was measured in a γ-counter (Wizard 3, PerkinElmer) and plotted as the amount of $^{111}$In-DTPA-pertuzumab (nmols bound) vs. the concentration of trastuzumab (nmol/L) using Prism® Ver. 4.0 software (GraphPad Inc., San Diego, CA). The assays were performed in duplicate and repeated in 3 separate experiments.

3.2.4 Effect of trastuzumab on binding of $^{111}$In-DTPA-pertuzumab in vitro

Approximately $5 \times 10^4$ SKBr-3 or $8 \times 10^4$ MDA-MB-361 cells were seeded in 24-well plates and cultured overnight. The medium was removed, and the cells incubated at 37 °C for 24, 48 or 72 h with 0 to 56 μg/mL of trastuzumab in 500 μL of fresh medium. The medium was again removed and the cells rinsed in cold PBS. The cells were then incubated with increasing concentrations (0 to 120 nmols/L) of $^{111}$In-DTPA-pertuzumab in serum-free medium for 3 h at 4 °C. The medium containing the unbound radioactivity was removed, the cells rinsed 2 times with PBS and then solubilized in 135 μL of 100 mmols/L of NaOH. The solubilized cells were transferred to γ-counting tubes and the cell-bound radioactivity measured in a γ-counter. The $B_{\text{max}}$ values were calculated by fitting a plot of cell-bound $^{111}$In-pertuzumab (nmols) vs. the concentration of unbound radioligand (nmols/L) to a 1-site saturation binding model using Prism® Ver. 4.0 software. $B_{\text{max}}$ values (nmols) were converted to the number of HER2 receptors/cell by counting the number of cells from control plates that were similarly seeded, treated with trastuzumab, and identically handled. Each assay was performed in duplicate and repeated in 3-6 separate experiments. Results are shown as the mean ± SD for all experiments.

3.2.5 Flow cytometry and confocal microscopic analysis of HER2 density

For flow cytometry, cells were seeded into T75 flasks (Sarstedt) and cultured overnight. They were then exposed to trastuzumab (14 μg/mL) in culture medium or to PBS in medium (control) for 72 h at 37 °C. The cells were rinsed twice with cold PBS and harvested. Fluorescein isothiocyanate (FITC)-conjugated anti-HER2/neu IgG1 (20 μL; Clone Neu 24.7, BD Biosciences, San Jose, CA), which binds to a different epitope than trastuzumab, was incubated for 45 min at 4 °C with 80 μL of a suspension of $1.3 \times 10^7$ cells/mL in ice cold Ca$^{2+}$ and Mg$^{2+}$-
free PBS (BD Biosciences) containing 0.2% bovine serum albumin (BSA) and 0.1% NaN3. Cells were recovered by centrifugation at 250 × g for 5 mins, rinsed twice with 1 mL of cold PBS and resuspended in 200 μL of 4% formaldehyde in PBS. Flow cytometry was performed using a FACScan (BD Biosciences) with 10,000 events recorded. Negative controls (no immunofluorescence staining) and isotype controls (FITC-mouse IgG1, eBioscience, San Diego, CA) were included. No non-specific binding was observed for the different cell lines or treatment conditions. Datasets were analyzed using CELLQuest software version 3.3 (BD Biosciences). Results were reported as the mean fluorescence intensity (MFI) calculated by subtracting the fluorescence intensity of the negative control from the fluorescence of cells stained for HER2/neu.

For confocal microscopy, 4-6 × 10^5 SKBr-3, MDA-MB-361 or MDA-MB-231 cells were seeded into chamber-slides (Thermo Fisher Scientific, Rochester, NY) and cultured overnight. The medium was removed and the cells incubated with trastuzumab (14 μg/mL) in 400 μL of fresh medium for 72 h at 37°C. Cells were rinsed three times with PBS and non-specific binding sites were blocked for 2 h with 3% BSA in Ca^{2+}- and Mg^{2+}-free PBS. Cells were rinsed once again with PBS and then incubated with FITC-anti-HER-2/neu IgG1 (BD Bioscience) for 1 h at RT. Following three rinses with PBS, the cells were fixed with 3.7% paraformaldehyde for 30 mins. The slides were mounted in Vectashield containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlington, ON) and kept at 4 °C overnight. Images were taken with an inverted LSM510 confocal microscope (Carl Zeiss, Toronto, ON) at the Advanced Optical Microscopy Facility (The Princess Margaret Hospital, Toronto, ON). Excitation was at 364 and 490 nm for visualization of DAPI and FITC, using 385- to 470-nm and 525-nm emission filters, respectively. Images were analyzed using LSM-Viewer software (version 3.5.0.376; Carl Zeiss).

3.2.6 Effects of trastuzumab on binding of ^111^In-DTPA-pertuzumab in vivo

Female athymic CD1 nu/nu mice (Charles River, Wilmington, MA) were implanted with a 0.72 mg, 60-day sustained release 17β-estradiol pellet (Innovative Research of America, Sarasota, FL). At least 24 h later, mice were inoculated subcutaneously (s.c.) with 1 × 10^7 MDA-MB-361 cells in 200 μL of a 1:1 mixture of Matrigel (BD Biosciences, Bedford, MA) and serum-free medium. After 2 weeks, groups of 3-4 mice with 70 ± 10 mm³ tumours (measured with external
calipers and calculated using the formula: \( length \times width^2 \times 0.5 \) were treated with 4 mg/kg of trastuzumab or non-specific human IgG (hIgG; Sigma Aldrich, Product I4506), or with PBS by intraperitoneal injection (i.p.) for 3 d. In a second study, groups of 3-4 tumour-bearing mice were treated with 4 mg/kg of trastuzumab, hIgG, anti-CD20 mAb, rituximab or PBS then 2 mg/kg weekly for 2 weeks. Following the 3 d or 3-week treatment courses, hIgG- and rituximab-treated mice were injected intravenously (i.v.) in the lateral tail vein with \(^{111}\text{In-DTPA-pertuzumab} \) (10 µg; 0.04-0.05 MBq/µg) in 200 µL of Sodium Chloride Injection USP. For trastuzumab- and PBS-treated mice, biodistribution studies were performed immediately following microSPECT/CT imaging at 72 h p.i. of \(^{111}\text{In-DTPA-pertuzumab} \) (10 µg; 1.3-3.1 MBq/µg). A group of 4 mice received a 100-fold excess of unlabeled pertuzumab 24 h prior to injection of \(^{111}\text{In-DTPA-pertuzumab} \) to evaluate the specificity of tumour uptake. At 72 p.i. of \(^{111}\text{In-DTPA-pertuzumab} \), mice were sacrificed and tumour, blood and normal tissues collected, weighed and their radioactivity measured in a \( \gamma \)-counter. Tumour and normal tissue uptake were expressed as mean ± SD percent injected dose/g (%ID/g) and as tumour/blood (T/B) and tumour/normal tissue (T/NT) ratios. The principles of Laboratory Animal Care (NIH Publication No. 86–23, revised 1985) were followed and all animal studies were conducted under a protocol (No. 989.5) approved by the Animal Care Committee at the University Health Network in accordance with Canadian Council on Animal Care (CCAC) guidelines.

### 3.2.7 MicroSPECT/CT imaging of tumour HER2 expression

MicroSPECT/CT imaging was performed at 72 h.p.i. of \(^{111}\text{In-DTPA-pertuzumab} \) (10 µg; 1.3-3.1 MBq/µg). Mice were anesthetized by inhalation of 2% isoflurane in O\(_2\). Imaging was performed on a NanoSPECT/CT tomograph (Bioscan, Washington, DC) equipped with 4 NaI detectors and fitted with 1.4 mm multi-pinhole collimators (FWHM ≤1.2 mm). A total of 24 projections were acquired into a 256 × 256 acquisition matrix with a minimum of 80,000 counts per projection. Images were reconstructed using an ordered subset expectation maximization (OSEM) algorithm (9 iterations). Quantification was performed by volume of interest (VOI) analysis using InvivoScope software version 1.34beta6 (Bioscan) and tumour and normal tissue uptake expressed as mean ± SD percent injected dose per voxel (%ID/voxel, 1 voxel=0.008 mm\(^3\)). Cone-beam CT images were acquired (180 projections, 1 s/projection, 45 kVp) prior to microSPECT acquisitions. Co-registration of microSPECT and CT images was performed using InvivoScope software.
3.2.8 Immunohistochemical (IHC) staining of explanted tumours

Excised tumours were cut into 2-3 mm sections, fixed in 10% neutral buffered formalin and paraffin embedded. Four µm-thick sections were dewaxed and rehydrated. Endogenous peroxidase and biotin activities were blocked using 3% H$_2$O$_2$ and an avidin/biotin blocking kit (Vector Laboratories), respectively. Sections were then blocked for 15 min with 10% normal serum and incubated with a mouse anti-c-erbB-2 monoclonal antibody (CB11; Novocastra Laboratories, Newcastle, UK) at a 1/100 dilution for 1 h, followed by an anti-mouse biotinylated secondary antibody (Vector Laboratories) for 30 mins and a horseradish peroxidase-conjugated ultrastreptavidin labeling reagent (ID Labs$^\text{TM}$ Inc, London, ON) for 30 mins at RT. After rinsing the sections twice with PBS, the reaction product was visualized using NovaRed solution (Vector Laboratories) and counterstained lightly with Mayer’s hemotoxylin. Sections were then dehydrated, cleared in xylene, and mounted in Permount (Fisher Scientific, Ottawa, ON). Negative control slides were prepared with omission of the primary anti-c-erbB-2 antibody. All samples were scored as the percentage of strong, complete, homogeneous membrane staining blindly by a pathologist (SD).

3.2.9 Statistical analysis

Comparisons of the specific binding of $^{111}$In-DTPA-pertuzumab to trastuzumab-exposed or control cells were made using a ratio t test (p<0.05). Tumour uptake of $^{111}$In-DTPA-pertuzumab in vivo was compared for different treatment groups by one-way parametric ANOVA using the Bonferroni correction for multiple comparisons (P<0.05). Correlations between biodistribution and imaging results were made using Pearson’s correlation test (P<0.05). All other statistical comparisons were made using Student’s t-test (P<0.05).

3.3 Results

3.3.1 $^{111}$In-DTPA-pertuzumab

Pertuzumab was substituted with 1.4 ± 0.3 DTPA molecules per IgG and labeled with $^{111}$In to a final RCP >97%. SE-HPLC analysis showed one major peak (retention time $[t_R] = 7.7$ min) corresponding to $^{111}$In-pertuzumab. The presence of aggregates or a higher molecular weight species was less than 10% ($t_R = 6.9$ mins). The presence of small molecular weight species, such
as DTPA, were less than 1.5% (t_r=13.0 mins). $^{111}$In-DTPA-pertuzumab demonstrated saturable binding to SKBr-3 cells with $K_d$ and $B_{\text{max}}$ values of $2.0 \pm 1.0 \text{ nmols/L}$ and $1.2 \pm 0.2 \times 10^6$ receptors/cell, respectively; these were comparable to values previously reported for SK-OV-3 cells (231).

3.3.2 Competition for HER2 binding on breast cancer cells

$^{111}$In-DTPA-pertuzumab binding to SKBr-3 or MDA-MB-361 cells was not displaced by increasing concentrations of trastuzumab up to 500 nmols/L, indicating that trastuzumab does not interfere with $^{111}$In-DTPA-pertuzumab binding to its epitope on HER2 (Fig. 3.1). The proportion of cell-bound radioactivity was greater for SKBr-3 cells than MDA-MB-361 cells due to their greater HER2 expression (330).

3.3.3 Effect of trastuzumab on binding of $^{111}$In-DTPA-pertuzumab in vitro

The saturation binding of $^{111}$In-DTPA-pertuzumab by SKBr-3 cells exposed to 14 µg/mL of trastuzumab at 37 °C was significantly reduced to 82 ± 6% (p=0.0094), 70 ± 10% (p=0.012) and 62 ± 2% (p=0.0001) compared to untreated cells at 24, 48 and 72 h, respectively (Fig. 3.2 A and Fig. 3.3 A). Higher or lower concentrations of trastuzumab (1, 3, 7, or 56 µg/mL) did not result in increased or decreased $^{111}$In-DTPA-pertuzumab binding compared to 14 µg/mL (P=0.68, data not shown). The effects of trastuzumab on binding of $^{111}$In-DTPA-pertuzumab were more profound for MDA-MB-361 than SKBr-3 cells. Exposure to trastuzumab (14 µg/mL) for 24, 48 or 72 h significantly reduced binding to 31 ± 13% (P=0.0012), 32 ± 13% (P=0.0009), and 32 ± 9% (P=0.0002), respectively, compared to control untreated cells (Fig. 3.2 B and Fig. 3.3 B). Rituximab or hIgG (14 µg/mL) did not significantly reduce $^{111}$In-DTPA-pertuzumab binding at 24, 48 or 72 h compared to untreated cells (p=0.13-0.96; Fig. 3.3 A and B).

3.3.4 Flow cytometry and confocal microscopic analysis of HER2 density

Flow cytometry and confocal microscopy showed that MDA-MB-231 cells with very low HER2 density exposed to PBS or to 14 µg/mL of trastuzumab for 72 h at 37 °C did not significantly bind FITC-conjugated anti-HER2/neu IgG1 antibodies (MFI 2.8 vs. 2.0, respectively; Fig. 3.4 A). In contrast, MDA-MB-361 or SKBr-3 cells with intermediate or high HER2 expression exposed to trastuzumab exhibited a 60% and 39% reduction, respectively in their binding of anti-HER2/neu antibodies compared to PBS-treated cells (MFI 26.2 vs. 66.2 and 66.0 vs. 107.4 respectively; Fig. 3.4 B and C). Confocal microscopy of MDA-MB-361 or SKBr-3 cells
exposed to PBS showed moderate or high HER2 membrane staining, respectively (Fig. 3.4 B and C). Exposure to trastuzumab markedly reduced membrane staining for HER2, especially for MDA-MB-361 but also for SKBr-3 cells.
**Fig. 3.1.** Displacement of the binding of $^{111}$In-DTPA-pertuzumab *in vitro* to SKBr-3 or MDA-MB-361 cells by increasing concentrations of trastuzumab. The higher binding of $^{111}$In-DTPA-pertuzumab to SKBr-3 compared to MDA-MB-361 cells is due to their greater HER2 expression. Data is presented from 1 representative assay and points represent the mean ± SD of duplicate determinations. The assay was repeated in 3 separate experiments.
**Fig. 3.2.** Representative *in vitro* saturation binding curves of $^{111}$In-DTPA-pertuzumab to SKBr-3 (A) or MDA-MB-361 (B) cells treated for 72 h with trastuzumab (14 µg/mL) and untreated controls. The $B_{\text{max}}$ values were calculated from the specific binding by fitting a plot of total cell-bound $^{111}$In-DTPA-pertuzumab (nmols) vs. the concentration of total added radioligand (nmols/L) to a 1-site saturation binding model using Prism® Ver. 4.0 software and then mathematically deriving the non-specific binding for subtraction from the total binding.
Fig. 3.3. Binding of $^{111}$In-DTPA-pertuzumab to (A) SKBr-3 and (B) MDA-MB-361 human breast cancer cells at selected times after incubation with 14 μg/mL of trastuzumab, rituximab or non-specific human IgG (hIgG). Values shown represent the mean ± SD of repeated experiments (n=3-6). Statistically significant differences compared to unexposed cells are shown (*P<0.05, **P<0.01, ***P<0.001)
Fig. 3.4. Confocal immunofluorescence microscopy (left and center panels) and flow cytometry (right panels) of (A) MDA-MB-231, (B) MDA-MB-361 and (C) SKBr-3 cells exposed to PBS (left) or 14 μg/mL trastuzumab (centre) for 72 h. HER2 was detected using a FITC-conjugated anti-HER-2/neu IgG1 (green) and the nucleus was counterstained with DAPI. For flow cytometry the mean fluorescence intensity (MFI) of trastuzumab-treated (T; bold line) and PBS-treated (P; dashed line) cells is indicated in the top right corner.
3.3.5 Effects of trastuzumab on binding of $^{111}$In-DTPA-pertuzumab in vivo

The tumour and normal tissue uptake of $^{111}$In-DTPA-pertuzumab and T/NT ratios at 72 p.i. in athymic mice bearing MDA-MB-361 xenografts are shown in Table 3.1. There was high tumour accumulation of radioactivity (34.6 ± 9.9 %ID/g) with a T/B ratio of 5.9 ± 1.4. Pre-injection of a 100-fold excess of unlabeled pertuzumab (1 mg) 24 h prior to $^{111}$In-DTPA-pertuzumab decreased tumour uptake 4.4-fold (7.8 ± 0.5 vs. 34.6 ± 9.9 %ID/g, respectively; P<0.01) and reduced the T/B ratio to 1.4 ± 0.3 (P<0.01), demonstrating that accumulation was HER2 specific. The highest normal tissue uptake of radioactivity was found in the kidneys, liver and spleen; these concentrations were similar to those previously reported for $^{111}$In-DTPA-trastuzumab (330). T/NT ratios were highest for muscle and lowest for the kidneys (Table 3.1).

Tumour uptake of $^{111}$In-DTPA-pertuzumab at 72 h p.i. was more than 2-fold lower in mice treated with trastuzumab (4 mg/kg) for 3 d than for mice receiving PBS (13.5 ± 2.6 vs. 28.5 ± 9.1 %ID/g, P<0.05; Fig. 3.5 A and Table 3.2). There also appeared to be decreased tumour accumulation of $^{111}$In-DTPA-pertuzumab in mice treated with hIgG compared to PBS-treated mice, but this was not statistically significant (21.8 ± 1.1 vs. 28.5 ± 9.1 %ID/g, P>0.05). A second study was performed to evaluate the ability of $^{111}$In-DTPA-pertuzumab to detect a therapeutic response to trastuzumab when administered at 4 mg/kg followed by weekly doses of 2 mg/kg for 2 weeks. Tumour accumulation of $^{111}$In-DTPA-pertuzumab was 4.5-fold lower in trastuzumab-treated mice compared to those treated with PBS (7.6 ± 0.4 vs. 34.6 ± 9.9 %ID/g, P<0.001; Fig. 3.5 B and Table 3.2). Again, there was not significantly diminished tumour uptake of $^{111}$In-DTPA-pertuzumab in mice treated with hIgG or rituximab compared to PBS-treated mice (25.0 ± 2.1 and 23.2 ± 5.3 %ID/g, respectively; P>0.05).
TABLE 3.1

Tumour and normal tissue distribution of radioactivity in athymic mice implanted subcutaneously with MDA-MB-361 human breast cancer xenografts at 72 hours post-injection of $^{111}$In-DTPA-pertuzumab

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Not Blocked *</th>
<th>Blocked</th>
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<tbody>
<tr>
<td></td>
<td>%ID/g</td>
<td>T/NT</td>
</tr>
<tr>
<td>Blood</td>
<td>5.9 ± 0.4</td>
<td>5.9 ± 1.4</td>
</tr>
<tr>
<td>Heart</td>
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<td>20.7 ± 6.4</td>
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<tr>
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<td>Spleen</td>
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<td>18.1 ± 6.4</td>
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<td>22.8 ± 7.5</td>
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<tr>
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<td>37.9 ± 9.5</td>
</tr>
<tr>
<td>Tumour</td>
<td>34.6 ± 9.9 †</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

* Groups of 3-4 tumour-bearing athymic mice were injected intravenously with $^{111}$In-DTPA-pertuzumab (10 µg) without (not blocked) or with (blocked) pre-injection of 1 mg of unlabeled pertuzumab.

† Statistically significant difference (P<0.01) compared to blocked group.
Fig. 3.5. Tumour uptake of $^{111}$In-DTPA-pertuzumab at 72 h p.i. in athymic mice bearing s.c. MDA-MB-361 xenografts at (A) 3 d post-treatment with PBS or 4 mg/kg of non-specific human IgG (hIgG) or trastuzumab or (B) 3 weeks post-treatment with PBS, rituximab, hIgG, or trastuzumab (loading dose of 4 mg/kg followed by weekly doses of 2 mg/kg). Significant differences compared to PBS treated mice are shown (*P<0.05, **P<0.001). Values shown represent the mean ± SD of replicate determinations (n=3-4).
TABLE 3.2

Tumour uptake (%ID/g) of $^{111}$In-DTPA-pertuzumab of individual athymic mice implanted subcutaneously with MDA-MB-361 human breast cancer xenografts at 72 hours post-injection

<table>
<thead>
<tr>
<th>Mouse</th>
<th>3-Day Treatment</th>
<th>3-Week Treatment</th>
</tr>
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<tr>
<td></td>
<td>PBS</td>
<td>Trastuzumab</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>32.5</td>
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<td>20.2</td>
<td>10.9</td>
</tr>
<tr>
<td>4</td>
<td>21.8</td>
<td>n/a</td>
</tr>
</tbody>
</table>

n/a: not available
3.3.6 MicroSPECT/CT imaging of tumour HER2 expression

MicroSPECT/CT images of athymic mice bearing MDA-MB-361 tumours treated with PBS at 72 h p.i. of $^{111}$In-DTPA-pertuzumab revealed strong tumour uptake; treatment with trastuzumab (4 mg/kg) for 3 d, however, diminished uptake considerably (Fig. 3.6 A and B). VOI analysis revealed a 2-fold reduction in tumour radioactivity compared to PBS-treated mice ($4.9 \pm 1.2 \times 10^{-5}\%\text{ID/voxel}$ vs. $1.0 \pm 0.35 \times 10^{-4}\%\text{ID/voxel}$, $P=0.05$; Fig. 3.6 E). Mice receiving PBS for 3 weeks showed very strong tumour uptake, but this was dramatically diminished in mice treated with trastuzumab (4 mg/kg followed by 2 mg/kg weekly; Fig. 3.6 C and D). Due to poor tumour signal, VOI analysis could not be reliably performed on mice treated with trastuzumab for 3 weeks. There was a strong, linear correlation between tumour VOI and biodistribution results ($r=0.97$, $P<0.0005$).

3.3.7 Immunohistochemical (IHC) staining of explanted tumours

HER2 downregulation was not apparent in explanted MDA-MB-361 tumours stained immunohistochemically with HER2/neu antibodies from mice treated with trastuzumab (4 mg/kg) for 3 days compared to PBS-treated mice (Fig. 3.7 and Table 3.3). H & E staining identified these sections as containing malignant cells. Explanted MDA-MB-361 tumours from mice treated for 3 weeks resulted in elimination of HER2 immunopositivity; this was associated with almost complete eradication of viable tumour cells (Fig. 3.7 and Table 3.3).
Fig. 3.6. Posterior whole-body microSPECT/CT images of representative athymic mice implanted s.c. in the right hind leg with MDA-MB-361 human breast cancer (BC) xenografts (white arrow) at 72 h post-injection of $^{111}$In-DTPA-pertuzumab. Mice were treated for 3 d with PBS (A) or 4 mg/kg of trastuzumab (B) or for 3 weeks with PBS (C) or 4 mg/kg followed by two weekly doses of 2 mg/kg of trastuzumab (D). Also visualized are the liver (small arrowhead) and kidneys (large arrowhead). There is a small superficial laceration on the back of the mouse in panel A which non-specifically accumulated radioactivity. Volume of interest (VOI) analysis (E) of the images in panels A and B showed a 52% decrease in tumour radioactivity post-treatment with trastuzumab (*$P=0.05$). VOI analysis was not performed on panel D due to the inability to clearly delineate the tumours in trastuzumab-treated mice.
**Fig. 3.7.** Immunohistochemical (IHC) staining (upper panels) using CB11 anti-HER2/neu antibodies of explanted representative MDA-MB-361 xenografts treated with PBS or trastuzumab for 3 days or 3 weeks. Bottom panels show H & E staining of immunostained sections.
### TABLE 3.3

Immunohistochemical analysis of explanted MDA-MB-361 human breast cancer xenografts stained for HER2

<table>
<thead>
<tr>
<th></th>
<th>PBS*</th>
<th>Trastuzumab 3 days*</th>
<th>Trastuzumab 3 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 ± 17</td>
<td>30 ± 13</td>
<td>n/a†</td>
</tr>
</tbody>
</table>

* Tumours were scored as the percentage of strong, complete membrane staining. Results are presented as the mean ± SD of 3 tumours.

† Not applicable. Too few viable tumour cells to assign a score.
3.4 Discussion

In this study, we showed for the first time that microSPECT/CT imaging with $^{111}$In-DTPA-pertuzumab sensitively detected changes in the concentration of HER2 in tumour tissue in s.c. BC xenografts in athymic mice caused by trastuzumab (Herceptin). Pertuzumab is a promising new immunotherapeutic agent for HER2-expressing BC, which aims to prevent dimerization with other HER family members; such dimerization is responsible for potent mitogenic signaling (38). Only one other group has reported the tumour and normal tissue distribution of pertuzumab, in this case $^{177}$Lu-pertuzumab in athymic mice bearing s.c. HER2-positive SK-OV-3 human ovarian cancer xenografts (231). In this earlier study, lower kidney, liver and spleen uptake were observed in comparison to $^{111}$In-DTPA-pertuzumab, possibly due to the higher in vivo stability of the radiometal complexed to the isothiocyanate-benzyl-CHX-A’-DTPA used.

A 2-fold decreased tumour uptake of $^{111}$In-DTPA-pertuzumab was measured in mice bearing MDA-MB-361 xenografts treated with trastuzumab (4 mg/kg) for 3 d compared to PBS-treated controls (Fig. 3.5 A). Decreased tumour radioactivity was also visualized by microSPECT/CT and quantified by VOI image analysis (Fig. 3.6 A, B and E). We interpret these findings as indicative of an early effect of trastuzumab in promoting HER2 downregulation, rather than reducing the number of viable HER2-positive tumour cells over this short time period. MDA-MB-361 cells exposed in vitro to trastuzumab (14 μg/mL) for only 24 h similarly exhibited a 70% reduction in the binding of $^{111}$In-DTPA-pertuzumab compared to untreated cells (Fig. 3.3 B). Interestingly, this same concentration of trastuzumab (14 μg/mL) only decreased the binding of $^{111}$In-DTPA-pertuzumab to SKBr-3 cells by a maximum of 38% at 72 h (Fig. 3.3 A). It was not possible to evaluate the effect of treatment with trastuzumab in vivo on the uptake of $^{111}$In-DTPA-pertuzumab in SKBr-3 cells, because these cells are poorly tumourigenic in athymic mice. The differences between MDA-MB-361 and SKBr-3 cells in HER2 downregulation caused by trastuzumab are consistent with earlier reports that HER2 on SKBr-3 cells are “internalization impaired” (110, 266). Hashizume et al. (266) proposed that co-existant HER3 on SKBr-3 cells may impair HER2 internalization, however, in our study, radioligand binding assays with $^{111}$In-DTPA-pertuzumab as well as flow cytometry and immunofluorescence confocal microscopy demonstrated that HER2 on MDA-MB-361 cells were downregulated by trastuzumab, despite the fact that these cells have similar HER3 expression as SKBr-3 cells (332). Interestingly, while hIgG and rituximab caused no changes in HER2 expression levels in SKBr-3 and MDA-MB-361 cells in vitro, there was an apparent, but
statistically insignificant decrease in tumour uptake of $^{111}$In-DTPA-pertuzumab mice treated with hIgG or rituximab in vivo. The reason for this phenomenon is not known.

HER2 downregulation is proposed as one means by which trastuzumab exerts its anti-tumour effects, but the relationship between tumour response in patients to the drug and its ability to diminish HER2 density is not proven (80). Trastuzumab-mediated HER2 downregulation has been reported in human BC cells in vitro (101, 103, 106), as well as in BC xenografts in mice in vivo (333, 334). However, Austin et al. (112) proposed that in SKBr-3 and BT-474 cells, there is continual recycling of HER2 from the cell surface to endosomes and back, and that HER2-bound trastuzumab accompanies these recycling receptors, but does not promote HER2 downregulation. A pilot clinical study aimed at identifying the mechanisms of action of trastuzumab in patients who were receiving the drug pre-operatively, found no significant change in tumour HER2 expression evaluated by IHC staining following treatment with 4 mg/kg followed by 3 weekly doses of 2 mg/kg (82). Others have reported changes in HER2 status only in small subsets of patients treated with trastuzumab (113, 114).

Possible explanations for these differing conclusions regarding trastuzumab-mediated HER2 downregulation are: i) variability in receptor downregulation between tumours in different patients or among different cell lines (as shown for SKBr-3 and MDA-MB-361 cells; Fig. 3.3), and ii) insensitivity in IHC staining for detection of changes in HER2 density, particularly when HER2 is heterogeneously downregulated within a lesion. HER2 3+ positivity is defined as uniform, intense membrane staining of >30% of tumour cells in a BC specimen (44). Thus, HER2 downregulation could occur in up to 70% of tumour cells and yet the specimen would still be designated as HER2 3+. In our study, there was significantly decreased uptake of $^{111}$In-DTPA-pertuzumab in MDA-MB-361 xenografts in mice treated with trastuzumab for 3 d (Fig. 3.5 A and 3.6 A and B), but no apparent decrease in HER2 positivity by IHC staining (Fig. 3.7). Kramer-Marek et al. also recently showed that IHC staining was insensitive to changes (up to 70 % reduction) in HER2 density of BT-474 BC xenografts in athymic mice caused by treatment with the heat shock protein-90 (Hsp90) inhibitor, 17-demethoxygeldanamycin (17-DMAG) (300). However, a 3-fold decreased tumour uptake of fluorine-18 labeled HER2 affibody ($^{18}$F-FBEM-ZHER2:342) was found by microPET and
biodistribution studies. Moreover, decreased HER2 was confirmed \textit{ex vivo} by Western blot and ELISA.

Smith-Jones et al. were the first to show that PET with $^{68}$Ga-labeled trastuzumab F(ab')$_2$ fragments could detect a 50% decrease in HER2 density in BT-474 xenografts in mice caused by treatment with the Hsp90 inhibitor, 17-allylaminogeldamycin (17-AAG) (234). Nonetheless, radiolabeled trastuzumab and its fragments are not optimal for imaging trastuzumab-mediated HER2 downregulation, because they bind to the same epitope, thus not able to differentiate between HER2 blocking by trastuzumab and receptor downregulation. Antibody probes such as $^{111}$In-DTPA-pertuzumab or affibodies (258, 300) that recognize epitopes on HER2 unique from that of trastuzumab can be used to differentiate these two processes. The faster blood clearance and higher T/B ratios of affibodies, antibody fragments or engineered antibody constructs may offer advantages over pertuzumab for imaging HER2 downregulation, especially in the clinical setting where same-day imaging may be achieved.

We also examined the tumour uptake of $^{111}$In-DTPA-pertuzumab at 72 h p.i. in athymic mice bearing MDA-MB-361 xenografts treated for 3 weeks with trastuzumab. In these studies, there was a 4.5-fold decrease in $^{111}$In-DTPA-pertuzumab uptake in MDA-MB-361 xenografts measured in biodistribution studies compared to mice treated with PBS (P<0.001). MicroSPECT/CT clearly identified tumours in the control mice receiving PBS but tumours in trastuzumab-treated mice were not visualized (Fig. 3.6 C and D). Immunohistochemical examination of the explanted tumours in trastuzumab-treated mice revealed a dramatic decrease in viable and HER2-positive tumour cells compared to PBS-treated mice (Fig. 3.7). In contrast, there was no apparent decrease in viable tumour cells in mice treated with trastuzumab for only 3 days compared to PBS-treated controls (Fig. 3.7). In patients, assessment of tumour viability changes for correlation with the uptake of $^{111}$In-DTPA-pertuzumab could potentially be assessed by PET using $^{18}$F-2-fluorodeoxyglucose ($^{18}$F-FDG) without the need for tissue sampling (182). Overall, our results demonstrated that microSPECT/CT imaging with $^{111}$In-DTPA-pertuzumab was powerful in its ability to detect a tumour response to trastuzumab over a longer time period, while visualizing an early molecular response (i.e. HER2 downregulation) shortly after commencing treatment with the drug.
3.5 Conclusion

In conclusion, microSPECT/CT imaging with $^{111}$In-DTPA-pertuzumab in athymic mice bearing s.c. MDA-MB-361 BC xenografts sensitively detected tumour HER2 downregulation associated with an early molecular response to trastuzumab as well as a longer term tumour response due to eradication of viable HER2-positive BC cells. IHC staining of explanted tumours was insufficiently sensitive to detect trastuzumab-mediated decreases in HER2 density. $^{111}$In-DTPA-pertuzumab may be valuable for imaging molecular and tumour response to trastuzumab in BC patients.
CHAPTER FOUR

$^{18}$F-FDG MicroPET/CT Differentiates Trastuzumab (Herceptin)-Responsive From Unresponsive Human Breast Cancer Xenografts in Athymic Mice

All experiments and analyses of data were carried out by Kristin McLarty except for the IHC scoring performed by Dr. Susan J. Done and the microCT imaging performed by Lisa DiDiodato and Rick Clarkson. MicroPET imaging was performed with technical assistance from Deborah A. Scollard, Douglass C. Vines and Dr. David E. Green.
4.0 Abstract

Purpose: Breast cancers (BC) with high HER2 expression are most likely to respond to trastuzumab (Herceptin®), however, the mechanisms of action of trastuzumab are complex and there are no established biomarkers to accurately monitor treatment outcome in individual patients. Therefore, our aim was to determine if there were any changes in 18F-FDG uptake in human BC xenografts in athymic mice treated with trastuzumab that were associated with response to the drug and which could have utility in monitoring response in patients.

Methods: Baseline tumour uptake of $^{18}$F-FDG was measured in mice with MDA-MB-361 HER2-overexpressing xenografts and MDA-MB-231 xenografts with very low HER2 expression by microPET imaging on Day 0. Mice were treated with PBS or trastuzumab (4 mg/kg) and microPET imaging was repeated 2 days post-treatment. Maintenance doses of trastuzumab (2 mg/kg) or PBS were administered on Days 7 and 14 and mice were imaged again on Days 9 and 16. Tumour uptake was measured as %ID/g by volume-of-interest analysis on Days 2, 9 and 16, followed by biodistribution studies on Day 16. Tumour growth was measured and a tumour growth index (TGI) was calculated.

Results: Trastuzumab treatment resulted in a significant decrease in tumour uptake of $^{18}$F-FDG in HER2-overexpressing MDA-MB-361 xenografts compared to PBS-treated control mice after 16 days of treatment (2.6 ± 0.8 vs. 4.6 ± 1.8 %ID/g, respectively; P<0.03), but not after 2 or 9 days of treatment (P=0.28-0.32). In contrast, there was no significant change in the tumour uptake of MDA-MB-231 xenografts with low HER2 expression during the entire course of therapy (4.4 ± 1.7 vs. 3.6 ± 1.1 %ID/g, respectively; P=0.31). Trastuzumab resulted in significant growth inhibition of MDA-MB-361 xenografts compared to PBS-treated controls as early as 10 days from initiation of treatment (TGI 0.7 ± 0.2 vs. 1.7 ± 0.3, respectively; P<0.0005), whereas no tumour growth inhibition was observed for MDA-MB-231 xenografts (5.3 ± 2.7 and 5.2 ± 3.0; P=0.95).

Conclusion: Changes in the tumour uptake of $^{18}$F-FDG post-therapy accurately identified responding and non-responding human breast cancer xenografts in athymic mice treated with trastuzumab (Herceptin®), however, diminished glucose utilization did not precede changes in tumour volume.
4.1 Introduction

Trastuzumab (Herceptin®, Genentech, Inc., South San Francisco, CA) is an immunotherapeutic agent for breast cancer (BC), which is directed against the human epidermal growth factor receptor 2 (HER2). HER2 is overexpressed in 20% of cases of BC and is a marker of aggressive disease and poor prognosis (26). Trastuzumab has been approved for the treatment of advanced BC and as adjuvant therapy for early-stage disease; it is also currently under investigation for use in neoadjuvant treatment of locally advanced BC (27, 335). Tumours with high HER2 expression are more likely to respond to trastuzumab-based therapy than HER2 low-expressing tumours (44). Only patients with HER2 amplification evaluated immunohistochemically in a primary tumour biopsy or identified by fluorescence in situ hybridization (FISH) probing for the HER2 gene are eligible for trastuzumab-based therapy (44). While these tests are useful in selecting patients who are most likely to respond to trastuzumab, there are no known clinical tumour markers that allow accurate prediction or monitoring of tumour response in an individual patient. Indeed, despite pre-selecting patients with HER2 overexpression, only a minority (12-35%) of patients respond to trastuzumab when administered as a single agent (120). Response rates improve when trastuzumab is combined with anthracyclines or taxanes, however approximately half of all eligible patients receive no therapeutic benefit while at risk for trastuzumab-associated cardiotoxicity (27, 336). The ability to monitor response early in the course of therapy would allow unresponsive patients to receive alternative therapy as well as reduce the cost to the healthcare system by discontinuing treatment in these patients (337).

Positron-emission tomography (PET) using \(^{18}\text{F}\)-fluoro-2-deoxy-D-glucose (\(^{18}\text{F}\)-FDG) reveals the increased glycolytic rate of malignant cells in tumours compared to surrounding normal tissues (338). The addition of computed tomography (CT) provides precise information on the anatomical location of \(^{18}\text{F}\)-FDG uptake and \(^{18}\text{F}\)-FDG PET/CT has proven useful in the detection and re-staging of recurrent BC and is under investigation as a probe for initial diagnosis and staging of primary disease (339, 340). An exciting application of FDG-PET is its ability to exploit treatment-mediated changes in tumour glucose utilization in cancer cells for monitoring response. Reductions in tumour uptake of FDG following chemotherapy correlate with response to neoadjuvant treatment of locally advanced BC (280-284), as well as to
chemotherapy of recurrent and metastatic disease (289, 290). $^{18}$F-FDG has also shown promise for predicting response of BC to hormonal therapy (196), and to treatment with the tyrosine kinase inhibitor, lapatinib (276). Additionally, a reduction in $^{18}$F-FDG-tumour uptake was observed following trastuzumab therapy in combination with the chemotherapeutic drugs, vinorelbine and gemcitabine, in case studies of individual patients with HER2-positive BC (341, 342). Our aim was, therefore, to examine any changes in $^{18}$F-FDG accumulation in human BC xenografts in athymic mice treated with trastuzumab, which may suggest that it could have utility in monitoring response to this drug in patients and would provide the needed preclinical evidence for a subsequent human study. We hypothesized that the decreased viability of BC cells exposed to trastuzumab may diminish their glucose utilization, which could then be sensitively detected by PET using $^{18}$F-FDG.

4.2 Materials and Methods

4.2.1 Breast cancer cells

MDA-MB-361 and MDA-MB-231 cells were purchased from the American Type Culture Collection (Manassas, VA). MDA-MB-361 cells were cultured in Leibovitz L15 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), under a 100% air atmosphere at 37ºC. MDA-MB-231 cells were cultured in Dulbecco’s minimal essential medium (Ontario Cancer Institute, Toronto, ON) supplemented with 10% FBS (Invitrogen) containing 100 U/mL penicillin and 100 µg/mL streptomycin, under a 5% CO$_2$ atmosphere at 37ºC.

4.2.2 Flow cytometry

We have previously quantified the HER2 expression level of MDA-MB-231 and MDA-MB-361 human BC cells, which display $5.4 \pm 0.7 \times 10^4$ and $5.1 \pm 1.7 \times 10^5$ receptors/cell, respectively (330). In this study, the relative HER2 expression level of MDA-MB-231 and MDA-MB-361 cells was verified by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated anti-HER2/neu IgG$_1$ (Clone Neu 24.7, BD Biosciences) as previously reported (343). Negative controls consisting of cells without immunofluorescence staining or cells incubated with the corresponding IgG isotype controls were included. Datasets were analyzed using CELLQuest software Ver. 3.3 (BD Biosciences). Results were reported as the mean fluorescence intensity (MFI) calculated by subtracting the fluorescence intensity of the isotype control from the fluorescence of cells stained for HER2/neu.
4.2.3 MicroPET imaging

MDA-MB-361 and MDA-MB-231 tumour xenografts were established in female athymic CD1 \textit{nu/nu} mice (Charles River, Wilmington, MA). For MDA-MB-361 xenografts, mice were first implanted with a 0.72 mg, 60-day sustained release 17β-estradiol intradermal pellet (Innovative Research of America, Sarasota, FL) at least 24 h prior to inoculation. The mice were inoculated subcutaneously (s.c.) in the left hind thigh with 1 \times 10^7 MDA-MB-361 cells and in the right hind thigh with 5 \times 10^6 MDA-MB-231 cells in 200 µL of a 1:1 mixture of Matrigel (BD Biosciences, Bedford, MA) and serum-free culture medium. Mice were randomized into treatment and control groups (n = 7) once tumours had reached an appropriate size (average tumour volumes were 113 ± 57 and 225 ± 156 mm$^3$ for MDA-MB-361 and MDA-MB-231 xenografts, respectively). All mice underwent microPET and microCT imaging prior to treatment (Day 0). Following these baseline images, mice were treated with PBS (control) or a loading dose of trastuzumab (4 mg/kg) diluted in PBS to a volume of 100 µL and administered intraperitoneally (i.p.). MicroPET and microCT imaging was repeated 2 days post-treatment. Maintenance doses of trastuzumab (2 mg/kg) or PBS were administered on Days 7 and 14 and mice were imaged again on Days 9 and 16. Trastuzumab doses were identical to those used clinically on a mg/kg basis (27). Images were acquired on a Focus 220 microPET (Siemens Preclinical Solutions, Knoxville, TN). All mice were fasted, with access to water, for 15-20 h prior to \textsuperscript{18}F-FDG administration and imaging. Mice were placed on a heating pad 1 h prior to injection and body weight was measured. Blood glucose levels were measured (Ascensia Contour, Bayer, Tarrytown, NY) by a needle prick to the saphenous or tail vein. Mice were anesthetized by inhalation of 2% isoflurane in oxygen and injected i.p. with 10.8 ± 1.2 MBq of \textsuperscript{18}F-FDG (Hamilton Health Sciences & McMaster University, Hamilton, ON). \textsuperscript{18}F-FDG was injected i.p. to facilitate repeat injections and reproducible uptake times in individual mice. Previous studies have shown that the tumour and normal tissue uptake of \textsuperscript{18}F-FDG administered by i.p. injection is comparable to the biodistribution of \textsuperscript{18}F-FDG following intravenous injection (i.v.) at 45-60 min post-injection (344). Following injection, the mice were maintained under anesthesia and warmed using a heating pad. Mice were imaged at exactly 60 min post-injection using an acquisition time of 10 mins. Images were reconstructed using an ordered subset expectation maximization (OSEM) followed by maximum \textit{a posteriori} probability (MAP)
reconstruction algorithm with no attenuation correction and no correction for partial volume effects as all tumours had diameters > 4 mm, which was greater than 2.5 times the full-width-at-half-maximum resolution of the tomograph (Siemens Preclinical Solutions). Quantification was performed by volume-of-interest (VOI) analysis using Inveon Research Workplace software (Siemens) and tumour uptake was expressed as the mean ± SD percent injected dose per gram (%ID/g) as well as the maximum percent injected dose per gram (%ID/gMAX), calculated using the maximum voxel value within the VOI. To calculate %ID, a quantification calibration factor was measured using a source of 18F with a known amount of radioactivity. Tumour volume was obtained by summing multiple 2-D regions of interest (ROIs) from consecutive tomographic planes encompassing the entire tumour volume. Volume was converted to mass by assuming the density of water (1 cm³ = 1 g). Immediately following microPET, microCT was performed on a GE eXplore Locus Ultra Preclinical CT Scanner (GE Healthcare, Chalfont St. Giles, UK) with routine acquisition parameters (80 kVp, 70 mA, voxel size of 150 μm × 150 μm × 150 μm). Co-registration of microPET and microCT images was performed using Inveon Research Workplace software (Siemens). MicroCT was used for anatomical reference, but not for delineating VOI’s. Response to trastuzumab was determined by measuring the tumours every 2-4 days using calipers and calculating tumour volume using the formula: \( \text{length} \times \text{width}^2 \times 0.5 \). A tumour growth index (TGI; mean ± SD) was calculated by dividing the tumour volume by the initial volume on Day 0 (330). The principles of Laboratory Animal Care (NIH Publication No. 86–23, revised 1985) were followed and all animal studies were conducted under a protocol (No. 989.5) approved by the Animal Care Committee at the University Health Network in accordance with Canadian Council on Animal Care (CCAC) guidelines.

### 4.2.4 Biodistribution studies

Correlative biodistribution studies were performed on the final day of microPET and microCT imaging (Day 16). Blood was collected via heart puncture at 59 min post-injection (p.i.) of 18F-FDG. Mice were immediately sacrificed at 60 min p.i. by cervical dislocation and imaged by microPET and microCT as described above. Following imaging, tumour and normal tissues were collected, weighed, and their radioactivity measured in a \( \gamma \)-counter (PerkinElmer, Wellesley, MA) Tumour and normal tissue uptake were expressed as mean ± SD percent injected dose per gram (%ID/g).
4.2.5 Immunohistochemical (IHC) staining of explanted tumours

*Ex vivo* IHC staining for HER2 and Ki67 expression was performed on explanted MDA-MB-361 and MDA-MB-231 tumours. Excised tumours were cut into 2-3 mm sections, fixed in 10% neutral buffered formalin for a minimum of 24 h and then paraffin embedded. A set of 4 µm-thick sections were dewaxed and rehydrated prior to antigen retrieval. Endogenous peroxidase and biotin activities were blocked respectively using 3% hydrogen peroxide and an avidin/biotin blocking kit (Lab Vision Cat# TA-015-BB). After blocking for 15 min with 10% normal horse serum, sections were incubated with the appropriate primary antibodies using previously optimized conditions. HER2 was detected using mouse anti-HER2 (CB11; 1/100 dilution for 1 h, Novocastra Laboratories Ltd., Newcastle, UK) and Ki67 was detected using mouse anti-Ki67 (MIB1; 1/100 dilution for overnight, Dako, Denmark). For HER2, the primary antibody was followed with a biotinylated horse anti-mouse secondary antibody (Vector labs, Burlingame, CA) for 30 min and horseradish peroxidase-conjugated ultrastreptavidin labeling reagent (ID labs™ Inc., London, ON, Canada) for 30 min. For Ki67, the primary antibody was followed with an anti-mouse polymer reagent (Vector ImmPress Kit). After rinsing 3 times with PBS, color development was done with freshly prepared NovaRed solution (Vector labs). Finally, sections were counterstained lightly with Mayer’s Hematoxylin, dehydrated in alcohols, cleared in xylene and mounted in Permount (Fisher Scientific, Pittsburgh, PA). Negative control slides were prepared without the primary antibody. All samples were scored blindly by a BC pathologist (SD). The HER2 sections were scored as the percentage of strong, complete, homogeneous membrane staining (mean ± SD). The Ki67 sections were scored as the percentage of positively staining nuclei (mean ± SD).

4.2.6 Statistical analysis

Comparisons of the tumour uptake of $^{18}$F-FDG and tumour growth indices were made using Student’s t-test (P<0.05). Correlation between the biodistribution and imaging results were determined using Pearson’s correlation test (P<0.05).
4.3 Results

4.3.1 Relative HER2 expression of breast cancer cells

The relative HER2 expression level of MDA-MB-361 and MDA-MB-231 cells was confirmed by flow cytometry (Fig. 4.1). MDA-MB-231 cells exhibited very low HER2 density, with a MFI of 3.0 (Fig. 4.1 A). In contrast, MDA-MB-361 cells exhibited greater HER2 expression as revealed by a MFI of 45.0 (Fig. 4.1 B). These flow cytometry results correlated very well with saturation radioligand binding assays performed with $^{111}$In-DTPA-trastuzumab, which demonstrated that MDA-MB-231 and MDA-MB-361 cells have $5.4 \pm 0.7 \times 10^4$ and $5.1 \pm 1.7 \times 10^5$ HER2 receptors/cell, respectively (330).

4.3.2 Tumour response to trastuzumab

Tumour growth was strongly inhibited by trastuzumab in mice bearing s.c. MDA-MB-361 xenografts. TGIs in trastuzumab-treated mice bearing these tumours were significantly lower than PBS-treated controls as early as 10 days following initiation of treatment (TGI $0.7 \pm 0.2$ vs. $1.7 \pm 0.3$, respectively; P<0.0005; Fig. 4.2 A). Following 16 days of treatment, PBS-treated MDA-MB-361 tumours had doubled in size compared to trastuzumab-treated tumours, which were reduced in volume by an average of 30% (TGI of $2.1 \pm 0.8$ vs. $0.7 \pm 0.2$, respectively; P<0.0005). There were no significant tumour growth inhibitory effects of trastuzumab on MDA-MB-231 tumours with much lower levels of HER2 (Fig. 4.2 B). The TGIs on Day 16 for PBS- and trastuzumab-treated mice bearing MDA-MB-231 xenografts were $5.3 \pm 2.7$ and $5.2 \pm 3.0$, respectively (P=0.95). Interestingly, MDA-MB-231 xenografts had a significantly greater proliferation rate than MDA-MB-361 xenografts (TGI on Day 16 of $5.3 \pm 2.7$ vs. $2.1 \pm 0.8$, respectively, P=0.01) despite the higher HER2 expression of the MDA-MB-361 tumours.
Fig. 4.1. Flow cytometric analysis of HER2 receptor expression of (A) MDA-MB-231 and (B) MDA-MB-361 human breast cancer cells. The mean fluorescence intensity (MFI) is indicated in the top right corner. Plots are representative results from 3 experiments.
Fig. 4.2. Tumour growth index of (A) MDA-MB-361 and (B) MDA-MB-231 xenografts. Mice were treated with a loading dose of trastuzumab (4 mg/kg) on Day 0, followed by maintenance doses (2 mg/kg) on Days 7 and 14. Significant differences compared to PBS treated mice are shown (*P<0.0005). Values shown are the mean ± SD (n=7).
4.3.3 MicroPET imaging and biodistribution studies

There was no difference in the tumour uptake of $^{18}$F-FDG in the trastuzumab-treated and PBS control groups of mice prior to treatment (baseline; Day 0) for both the MDA-MB-361 and MDA-MB-231 xenografts (Fig. 4.3 A and B). Interestingly however, average baseline (pretreatment) tumour uptake of $^{18}$F-FDG was marginally greater for MDA-MB-231 xenografts compared to MDA-MB-361 xenografts (5.4 ± 1.7 vs. 4.1 ± 1.4 %ID/g, respectively; P=0.049), but maximum baseline tumour uptake was significantly greater for MDA-MB-231 xenografts compared to MDA-MB-361 xenografts (10.7 ± 3.1 vs. 6.4 ± 1.8 %ID/g$_{MAX}$, respectively; P=0.0003; Fig. 4.3 C).

There was no significant change in the tumour uptake of $^{18}$F-FDG in MDA-MB-361 xenografts following 2 or 9 days of trastuzumab treatment compared to baseline or PBS-treated controls (P=0.28-0.32; Fig. 4.3 A). After 16 days of trastuzumab therapy, however, there was a significant 43% decrease in the mean tumour uptake of $^{18}$F-FDG in trastuzumab-treated mice compared to PBS-treated control mice bearing HER2 overexpressing MDA-MB-361 xenografts quantified by VOI analysis (4.6 ± 1.8 vs. 2.6 ± 0.8 %ID/g, respectively; P<0.03; Fig. 4.3 A). Similarly, there was a 60% reduction in tumour uptake of $^{18}$F-FDG measured by biodistribution studies (5.1 ± 2.1 vs. 2.1 ± 0.9 %ID/g, respectively; P<0.002; Fig. 4.3 A). Furthermore, on Day 16, tumour uptake of $^{18}$F-FDG was 35% lower than the tumour uptake at baseline in trastuzumab-treated mice bearing MDA-MB-361 xenografts (2.6 ± 0.8 vs. 4.0 ± 0.7 %ID/g, respectively P=0.01; Fig. 4.3 A and 4.4 A). A similar trend was observed in the maximum tumour uptake ($\%$ID/g$_{MAX}$), in which trastuzumab treatment resulted in a 55% decrease in %ID/g$_{MAX}$ compared to PBS-treated tumours and a 34% decrease compared to pre-treatment $^{18}$F-FDG uptake (P<0.03; Fig. 4.3 C). In contrast, there was no significant difference in the mean or maximum tumour uptake of $^{18}$F-FDG in MDA-MB-231 xenografts in trastuzumab treated mice compared to PBS-treated controls (P=0.31-74; Fig. 4.3 B, C and Fig. 4.4 B). Interestingly, there was a modest, but statistically insignificant decrease in the average tumour uptake of $^{18}$F-FDG in MDA-MB-231 xenografts in trastuzumab- and PBS-treated mice compared to baseline uptake over the course of therapy (Fig. 4.3 B). This reduction in tumour uptake was only statistically significant for PBS-treated control mice on Day 16 compared to baseline (3.6 ± 1.1 vs. 5.7 ± 1.6, respectively P=0.01). Nonetheless, there was no difference in
the maximum tumour uptake (%ID/g\textsubscript{MAX}) for trastuzumab- and PBS-treated mice compared to baseline uptake (Fig. 4.3 C). Additionally, areas of central necrosis were visualized on many of the MDA-MB-231 tumours (Fig. 4.4 C).

An excellent correlation was observed between VOI analysis of the microPET images and tumour uptake measured \textit{ex vivo} in biodistribution studies on Day 16 (r=0.94, P<0.0001; Fig. 4.5 A). Furthermore, there was an excellent linear correlation between the tumour volume measured by VOI analysis (mm\textsuperscript{3}) and tumour weights (mg) obtained at the end of the study (r=0.99, P<0.0001; Fig. 4.5 B). There were no decreases in body mass among the mice during therapy and differences in body mass between mice were negligible (22.3 ± 1.6 g). Additionally, blood glucose levels of fasted mice were consistent over the 16 days with an average of 6.4 ± 1.4 mmol/L.

4.3.4 Immunohistochemical (IHC) staining of explanted tissues

PBS-treated control MDA-MB-361 xenografts stained strongly for HER2, with an average of 57 ± 20 % positively staining cells (Table 4.1 and Fig. 4.6 A). Following trastuzumab therapy, there was a significant decrease in HER2 staining due to a dramatic eradication of viable tumour cells (Fig. 4.6 A). Additionally, there was an apparent, but statistically insignificant reduction in the percentage of positively staining cells following trastuzumab treatment compared to PBS-treated controls (20 ± 17 % vs. 57 ± 20 %; P=0.08), however, these results should be considered cautiously as there were very few viable cells remaining following treatment (Fig. 4.6 A). PBS-treated MDA-MB-231 tumours were negative for HER2 expression and no changes in HER2 density were noted following trastuzumab therapy (Table 4.1 and Fig. 4.6 B). The percentage of Ki67 positive cells in MDA-MB-361 tumours in control mice receiving PBS was 20 ± 10 %, which was unchanged following trastuzumab therapy (23 ± 10 %; Table 4.1 and Fig. 4.6 A). In contrast, in the rapidly proliferating MDA-MB-231 tumours in PBS-treated control mice 85 ± 7 % of cells were Ki67 positive, which was similarly unchanged with trastuzumab exposure (90 ± 0 %; Table 4.1 and Fig. 4.6 B).
Fig. 4.3. Tumour uptake of $^{18}$F-FDG measured by volume-of-interest (VOI) analysis and biodistribution studies in athymic mice bearing MDA-MB-361 (A) and MDA-MB-231 human BC xenografts (B). Pre-treatment uptake of $^{18}$F-FDG was measured by microPET/CT on Day 0, followed by post-treatment scans on Days 2, 9 and 16. Biodistribution studies were performed on Day 16 following microPET/CT imaging. Significant differences compared to PBS treated mice are shown (*P<0.03, **P<0.002). Data is presented as the average percent injected dose per gram (%ID/g; mean ± SD, n=5-7). The maximum %ID/g ($%ID/g_{\text{MAX}}$) of MDA-MB-361 and MDA-MB-231 xenografts before and after 16 days of treatment with PBS and trastuzumab (C) was calculated from the voxel with the highest accumulation of $^{18}$F-FDG. Significant differences compared to PBS-treated mice and baseline uptake are shown (*P<0.03).
Fig. 4.4. Coronal (upper) and transaxial (lower) microPET co-registered microCT images of representative athymic mice co-implanted s.c. in the left hind leg with MDA-MB-361 xenografts and right hind leg with MDA-MB-231 human BC xenografts (A) or with only MDA-MB-231 xenografts on the right hind leg (B) at 60 min post-intraperitoneal injection of $^{18}$F-FDG. MicroPET/CT images were acquired pre-treatment on Day 0 (I) or post-treatment on Day 16 (II) of trastuzumab therapy. Tumours of interest are indicated by white solid arrows (MDA-MB-361) or broken arrows (MDA-MB-231). Also shown is a representative large MDA-MB-231 xenograft with extensive central necrosis (C). Image intensities were adjusted for optimal delineation of volumes-of-interest and not for equivalent visualization.
**Fig. 4.5.** Correlations between estimation of $^{18}$F-FDG concentrations by volume-of-interest analysis of images and tissue biodistribution studies (A) as well as tumour volume measured by volume-of-interest analysis and tumour mass of explanted xenografts (B).
TABLE 4.1
Immunohistochemical analysis of explanted MDA-MB-361 and MDA-MB-231 human breast cancer xenografts stained for HER2 and Ki67*

<table>
<thead>
<tr>
<th></th>
<th>MDA-MB-361</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS Trastuzumab†</td>
<td>PBS Trastuzumab</td>
</tr>
<tr>
<td>HER2‡</td>
<td>57 ± 20</td>
<td>20 ± 17</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Ki67§</td>
<td>20 ± 10</td>
<td>23 ± 10</td>
</tr>
<tr>
<td></td>
<td>85 ± 7</td>
<td>90 ± 0</td>
</tr>
</tbody>
</table>

* Results are presented as the mean ± SD of 3 tumours.
† Following trastuzumab treatment, there were very few tumour cells remaining for analysis.
‡ Tumours were scored as the percentage of strong, complete membrane staining.
§ Tumours were scored as the percentage of positively staining nuclei.
**Fig. 4.6.** Immunohistochemical (IHC) staining using CB11 anti-HER2/neu, and MIB-1 anti-Ki67 antibodies of explanted representative MDA-MB-361 xenografts (A) and MDA-MB-231 xenografts (B) treated for 16 days with PBS (upper panels) or trastuzumab (lower panels). The MDA-MB-361 control tumours treated with PBS demonstrated positive membrane staining for HER2. In MDA-MB-361 tumours, there was an apparent reduction in HER2 staining following treatment with trastuzumab. In contrast, the percentage of Ki67-positive cells was unchanged following treatment. Following treatment with trastuzumab, there was a significant reduction in the number of viable tumour cells in MDA-MB-361 xenografts. MDA-MB-231 xenografts treated with PBS were negative for HER2, but strongly positive for Ki67. No changes were observed following treatment with trastuzumab in MDA-MB-231 tumours.
4.4 Discussion

In this study we demonstrated that monitoring changes in tumour glucose utilization by microPET/CT using $^{18}$F-FDG post-therapy differentiated trastuzumab-responding and non-responding human BC xenografts in athymic mice. Tumour uptake of $^{18}$F-FDG measured by volume-of-interest (VOI) analysis was reduced by 43% in athymic mice bearing trastuzumab-sensitive and HER2-overexpressing MDA-MB-361 xenografts compared to PBS-treated controls (P<0.03; Fig. 4.3 A). Similar results were observed by biodistribution studies, in which a 60% reduction in tumour uptake of $^{18}$F-FDG was observed in mice with MDA-MB-361 tumours receiving trastuzumab compared to control mice receiving PBS (P<0.002; Fig. 4.3 A). Furthermore, there was a significant 35% reduction in the tumour uptake of $^{18}$F-FDG in trastuzumab-treated mice compared to baseline $^{18}$F-FDG uptake in these same mice evaluated prior to treatment (P=0.01; Fig. 4.3 A). This reduction in tumour accumulation of $^{18}$F-FDG correlated with the caliper measured tumour response to trastuzumab. Significant tumour growth inhibition was observed over the course of 16 days of trastuzumab therapy resulting in a 30% reduction in tumour volume compared to PBS-treated MDA-MB-361 xenografts which doubled in size (P<0.0005; Fig. 4.2 A). In contrast, there was no reduction in uptake of $^{18}$F-FDG in non-responding MDA-MB-231 xenografts in mice treated with trastuzumab (Fig. 4.3 B). In accordance with the $^{18}$F-FDG PET results, no significant growth inhibition of MDA-MB-231 xenografts was observed during trastuzumab therapy compared to PBS-treated controls (Fig. 4.2 B).

There was only a marginal difference in the average baseline tumour uptake of $^{18}$F-FDG in both xenografts (Fig. 4.3 A and B), despite the significantly greater proliferation rate of the MDA-MB-231 xenografts shown by the tumour growth index (Fig. 4.2 A and B) and Ki67 staining of explanted tissue (Fig. 4.6 A and B). This discrepancy may be due to the large areas of necrosis evident in some of the MDA-MB-231 xenografts (Fig. 4.4 C). Areas of necrosis would not be expected to accumulate $^{18}$F-FDG and, as a result, lower the average tumour uptake of $^{18}$F-FDG (%ID/g). In contrast, the maximum baseline tumour uptake (%ID/g$_{\text{MAX}}$) was significantly greater for the MDA-MB-231 xenografts compared to the MDA-MB-361 xenografts (P=0.01; Fig. 4.3 C), consistent with regions of rapidly proliferating cells in the MDA-MB-231 tumours. In MDA-MB-361 xenografts, the similar maximum (%ID/g$_{\text{MAX}}$) and
average (%ID/g\text{AVG}) values demonstrates that uptake in these tumours was reasonably homogenous, whereas the uptake in some of the MDA-MB-231 tumours was much higher at the periphery of the tumour (Fig. 4.4 C). The average tumour uptake of $^{18}$F-FDG also decreased in the MDA-MB-231 xenografts over the 16-day therapy in both the PBS- and trastuzumab-treated mice (Fig. 4.3 B). This is possibly explained by the greater proportion of the MDA-MB-231 xenografts that became necrotic as the tumours grew larger, resulting in a decrease in the average %ID/g over the course of treatment. This observation demonstrates that average tumour uptake of $^{18}$F-FDG may not be representative of the proliferative capacity of a tumour if large areas of necrosis are present. In this case, the maximum tumour uptake may be a more powerful prognostic factor. Indeed, both average and maximum standardized uptake values ($\text{SUV}_{\text{AVG}}$ and $\text{SUV}_{\text{MAX}}$) are used clinically (285). Standard uptake values are normalized for body weight, body surface area, or lean body mass (345). This correction is less critical for preclinical studies, because differences in body mass or surface area of mice are usually insignificant. In our study, differences in body mass between mice were negligible (22.3 ± 1.6 g). Furthermore, no correction was applied for differences in blood glucose levels, as fluctuations in blood glucose levels were minimal and these corrections have not been shown to minimize variability in preclinical studies (346). Previous studies have reported the effects of fasting times, anesthetic agents, and blood glucose levels on the uptake of $^{18}$F-FDG in mice (344, 346, 347). In order to minimize variability, all mice were handled under the same fasting state, anesthetic agent, incubation time, and body temperature.

To date, no clinical studies have systemically evaluated the use of $^{18}$F-FDG PET for monitoring response to trastuzumab in HER2-positive BC, however, two case studies reported decreased tumour uptake of $^{18}$F-FDG in patients responding to trastuzumab in combination with vinorelbine and gemcitabine (341, 342). Additionally, a small clinical study by Kawada and co-workers has shown that monitoring tumour glucose utilization by $^{18}$F-FDG PET may be predictive of response to lapatinib, a dual kinase inhibitor of EGFR and HER2 (276). In this study, a patient with a partial response had a 60% decreased tumour uptake ($\text{SUV}_{\text{MAX}}$) of $^{18}$F-FDG, while in patients with stable disease, uptake of the radiopharmaceutical decreased by 6-42% one month after the initiation of therapy. Patients whose disease progressed had an increase in $^{18}$F-FDG uptake in 2 of 3 cases. These studies, demonstrate that $^{18}$F-FDG PET could potentially be a powerful tool for monitoring response of tumours in patients to treatment with molecularly targeted therapies such as trastuzumab (Herceptin®) and selective tyrosine kinase inhibitors. Indeed, it has been proposed that $^{18}$F-FDG PET may provide an earlier indication of
response and resistance than radiographical imaging, which relies on changes in tumour size. In the study by Kawada and co-workers, changes in tumour uptake of $^{18}$F-FDG PET in one patient were apparent 2 months before tumour progression was confirmed by CT (276). In contrast, a preclinical study by Shah and co-workers found no statistically significant changes in the tumor uptake of $^{18}$F-FDG in HER2-overexpressing BT-474 breast cancer xenografts or MMTV/HER2 tumors following 1-3 weeks of trastuzumab therapy (348). Interestingly, in our study there was a 31% reduction in the tumour uptake of $^{18}$F-FDG in MDA-MB-361 HER2-overexpressing xenografts after 9 days of treatment, however, this difference was not statistically significant ($P=0.32$; **Fig. 4.3 A**). A significant decrease in tumour uptake of $^{18}$F-FDG was only detected following 16 days of treatment. Nevertheless, a significant difference in the volume of PBS- and trastuzumab-treated tumours was apparent after only 10 days of treatment (**Fig. 4.2 A**). This suggests that the trastuzumab-mediated reduction in tumour uptake of $^{18}$F-FDG is likely due to a reduction in the total number of viable tumour cells, rather than decreased glucose utilization by individual cells. However, a significant reduction in the %ID/$g_{\text{MAX}}$ of MDA-MB-361 xenografts following treatment with trastuzumab, also suggests reduced glucose utilization of the remaining viable cells. Thus, while $^{18}$F-FDG PET sensitively differentiated post-therapy between trastuzumab responsive and resistant BC xenografts, it did not provide an earlier indication of response to trastuzumab than anatomical changes measured using calipers. However, due to the lack of soft tissue contrast, CT was not used in this study as is commonly done in patients and therefore, comparison of the temporal sensitivity $^{18}$F-FDG PET and CT for detection of tumor response was not possible. Moreover, $^{18}$F-FDG PET was not performed between 9 and 16 days of treatment, and thus it is not known precisely when statistically significant changes could have been detected during this time interval.

In this study, expression levels of HER2 and Ki67 were examined in explanted tumour tissue from PBS- and trastuzumab-treated mice. HER2 expression levels previously measured in saturation radioligand binding assays using $^{111}$In-labeled trastuzumab (330) corresponded well with *in vitro* flow cytometry analysis of the cell lines. IHC analysis of explanted tumour tissue showed that the MDA-MB-361 xenografts overexpressed HER2, which appeared to be reduced by approximately 65% in trastuzumab treated xenografts compared to PBS-treated controls ($P=0.08$), however, few viable cells were available for analysis in trastuzumab-treated
xenografts. IHC analysis of MDA-MB-231 xenografts confirmed that these tumours were negative for HER2 and the expression was unchanged following trastuzumab treatment. Staining of the proliferation marker Ki67 was in agreement with the growth index and maximum $^{18}$F-FDG accumulation of the tumours. In the MDA-MB-361 tumours, $20 \pm 10\%$ of the cells were positive for Ki67 expression and these tumours demonstrated modest growth, resulting in a 2-fold increase in the tumour volume of PBS-treated mice and a maximum uptake of $^{18}$F-FDG of 6.2-8.8 %ID/g$_{\text{MAX}}$ over the course of therapy. In the MDA-MB-231 xenografts, a greater proportion of cells were positive for Ki67 expression ($85 \pm 7\%$), which was reflected in the 5-fold increase in the tumour volume of PBS-treated control mice over 16 days and maximum tumour uptake of $^{18}$F-FDG of 10.2-12.2 %ID/g$_{\text{MAX}}$. These results suggest that tumour uptake of $^{18}$F-FDG is reflective of the level of proliferation of the tumour. Clinical studies have also found a positive correlation between Ki67 staining and uptake of $^{18}$F-FDG in BC (349-352), although this relationship is not well defined for lobular carcinoma, which has lower uptake of $^{18}$F-FDG than ductal carcinoma (349-351, 353). Clinically, most data suggest there is no relationship between HER2 expression evaluated by IHC and tumour uptake of $^{18}$F-FDG (350-352, 354, 355). Only Ueda and co-workers found a positive correlation between expression of HER2 and tumour uptake of $^{18}$F-FDG (353). Our results are consistent with these reports in that MDA-MB-231 xenografts with low HER2 density had higher uptake of $^{18}$F-FDG than MDA-MB-361 xenografts, which overexpress HER2.

While this study provides proof-of-principle that $^{18}$F-FDG can differentiate post-therapy between trastuzumab-responsive and resistant BC xenografts, the sensitivity of $^{18}$F-FDG PET for tumours which are only partially responsive to trastuzumab or those that acquire resistance is unknown and warrants further investigation. Furthermore, other molecular imaging agents may also be useful for monitoring response to trastuzumab. In a previous study, we have shown that HER2 downregulation – one of the proposed mechanisms of action of trastuzumab – could be sensitively detected by microSPECT/CT imaging using $^{111}$In-DTPA-pertuzumab following only 3 days of trastuzumab therapy in MDA-MB-361 xenografts (343). Furthermore, changes in tumour uptake of $^{111}$In-DTPA-pertuzumab were informative on the decreased viability of tumour cells following 3 weeks of trastuzumab therapy. Additionally, 3’-deoxy-3’-[$^{18}$F]fluorothymidine ($^{18}$F-FLT), which accumulates in tumours in proportion to their proliferative properties and has shown promise in identifying early responding and nonresponding tumours in patients receiving chemotherapy may be useful for monitoring response to trastuzumab (296, 348).
4.5 Conclusion

We conclude that changes in tumour uptake of $^{18}$F-FDG post-therapy accurately differentiated responding and non-responding human BC xenografts in athymic mice treated with trastuzumab (Herceptin). $^{18}$F-FDG PET for monitoring response to trastuzumab therapy warrants further investigation to determine if changes in tumour uptake of $^{18}$F-FDG may occur prior to anatomical changes measured by radiographic imaging and if such imaging could detect the emergence of trastuzumab resistance in previously responsive tumours.
CHAPTER FIVE

Overall Thesis Conclusions and Future Research
5.1 Thesis Conclusions

The overall conclusions of this thesis are:

1) There is a non-linear correlation between tumour uptake of $^{111}\text{In}$-DTPA-trastuzumab and HER2 expression when tumour uptake is corrected for non-specific IgG tumour localization and/or circulating radioactivity. There is, however, no direct correlation between HER2 expression levels of human breast cancer xenografts and the uncorrected tumour uptake of $^{111}\text{In}$-DTPA-trastuzumab in athymic mice.

2) The uncorrected tumour uptake of $^{111}\text{In}$-DTPA-trastuzumab was associated with response to treatment with trastuzumab (Herceptin®) in the limited panel of tumour xenografts examined, but it is important to recognize that compensatory trastuzumab-resistance mechanisms can cause discordance between HER2 density and response to the drug.

3) MicroSPECT/CT imaging with $^{111}\text{In}$-DTPA-pertuzumab in athymic mice bearing human breast cancer xenografts sensitively detected tumour HER2 downregulation associated with an early molecular response to trastuzumab as well as a longer term tumour response due to eradication of viable HER2-positive BC cells.

4) Changes in tumour glucose utilization measured by PET imaging using $^{18}\text{F}$-FDG accurately identified responding and non-responding human breast cancer xenografts with different HER2 expression in athymic mice treated with trastuzumab. PET with $^{18}\text{F}$-FDG may be a promising tool for prediction of response to trastuzumab for HER2-positive breast cancer.
5.2 Discussion

5.2.1 The clinical role of imaging HER2 expression in breast cancer

HER2 overexpression occurs in approximately 1 in 4 cases of BC, therefore molecular imaging of HER2 overexpression is not suitable for BC screening, as the majority of BCs would not be detected by nuclear medicine imaging of HER2. Currently mammography is the only imaging modality for screening of BC that is proven to reduce mortality. Mammography and ultrasound are the most commonly used imaging modalities for the diagnosis and staging of BC, with MRI and nuclear medicine being increasingly used for mammographically indeterminant findings, detection of recurrent disease, detection in radiographically dense breasts, as well as for determining the extent of disease prior to surgery (see section 1.6). The increasing number of molecularly targeted cancer therapeutics, however, has created a need for new tools to guide the use of these drugs which are likely to be effective only in subsets of patients due to heterogeneity in the expression of the target genes and proteins. In the case of trastuzumab, patients with HER2 overexpression are selected by FISH and IHC analysis of gene amplification and protein overexpression, respectively (discussed in section 1.2.4.2). The accuracy of these methods for selecting patients with HER2 overexpression are comparable, however both are limited by sampling errors due to tumour heterogeneity and to a small extent by discordance between primary tumours and metastases (356). Accuracy of IHC can also be limited by differences in sample handling and processing, as well as misinterpretation and variable interobserver reproducibility especially in equivocal cases (356). Additionally, IHC staining for HER2 using antibodies reactive only with the intracellular domain (e.g. CB11 or HercepTest) cannot distinguish between full length HER2 and the truncated receptor (p95) due to proteolytic cleavage of the ECD (357). The truncated phenotype may stain strongly by IHC, but the receptors are no longer able to bind trastuzumab, causing one form of resistance (89, 90). Therefore, patients with high levels of p95 may not respond well to trastuzumab therapy. The ASCO and CAP expert panel reported that approximately 20% of current HER2 test results may be inaccurate (44). Molecular imaging of HER2 may be useful in addition to IHC and FISH in selecting patients for treatment with antiHER2 therapies. Molecular imaging has the advantage of visualizing total HER2 expression (primary and metastasis) in a single scan and providing information about delivery of drugs to target tissues. Additionally, functional imaging of HER2 may be useful in monitoring response to therapy, such as trastuzumab. I have shown in Chapter
3, that $^{111}$In-DTPA-pertuzumab sensitively detects early changes in HER2 expression levels in MDA-MB-361 human BC xenografts following treatment with trastuzumab, as well as late changes in HER2 tissue concentration due to a therapeutic response and reduction in HER2-expressing tumour cells. Furthermore, these early changes in HER2 expression could be detected after only 3 days of treatment, much sooner than changes in tumour uptake of $^{18}$F-FDG were detected in MDA-MB-361 xenografts treated with the same dose of trastuzumab (Chapter 4). A significant change in tumour uptake of $^{18}$F-FDG was detectable by PET imaging only after 16 days. Other groups have also shown that radiolabeled antibody fragments and affibodies can detect HER2 downregulation 1-3 days following treatment with Hsp90 inhibitors, another class of antiHER2 therapeutic agents currently being investigated in Phase II trials for MBC (234, 300, 358). While trastuzumab likely induces HER2 downregulation through internalization and degradation of cell surface receptors, Hsp90 inhibitors interfere with the Hsp90 chaperone protein which mediates proper folding and maturation of HER2 protecting newly formed receptors from routing to the proteosome for degradation, as well as permitting recycling of internalized receptors back to the cell surface (112, 359). Thus, Hsp90 inhibitors such as 17-AAG and 17-DMAG block the function of Hsp90, routing intracellular receptors to the proteosome for degradation. Therefore combination therapy of trastuzumab and Hsp90 inhibitors could be complementary (360). Recently, a phase I trial examined this combination therapy with promising results (299). Molecular imaging of HER2 downregulation using $^{111}$In-DTPA-pertuzumab, as shown in Chapter 3, may provide a sensitive means of evaluating tumour response in situ to this combination therapy. While, it has been shown preclinically that treatment-mediated changes in HER2 expression can be sensitively and non-invasively detected by SPECT and PET, the relationship between changes in HER2 tissue concentration and therapeutic response warrants further investigation.

The value of HER2 imaging agents in the management of cancer will likely extend beyond BC. Targeted HER2 therapeutics are being evaluated in a variety of malignancies in which subsets of patients overexpress HER2, such as in advanced urothelial cancer (361), CRC (362), prostate cancer (363), ovarian cancer (364) and NSCLC (276).
5.2.2 Quantifying HER2 expression in vivo

In vivo characterization of HER2 expression levels by molecular imaging may be a powerful tool in addition to IHC and FISH for selecting patients for HER2 therapy and monitoring response to treatment, however, various challenges must be addressed. A critical aspect of defining HER2-positivity by molecular imaging is the relationship between tumour uptake of HER2 imaging agents and tumour HER2 expression level (receptors per cell), however, little work has been done to quantify this relationship, and more importantly, the relationship between tumour uptake of the imaging probes, HER2 density and therapeutic outcome. Many preclinical studies have shown the specificity of HER2 imaging probes demonstrating high tumour uptake in HER2-overexpressing BC xenografts and low uptake in xenografts with low or no HER2 expression (see section 1.8). Few studies however, have attempted to define tumour uptake in relation to HER2 density (receptors per cell) or clinically relevant assays such as IHC and FISH. In Chapter 2 I found a disparity between the tumour uptake of $^{111}$In-DTPA-trastuzumab and HER2 expression levels of human BC xenografts measured in vitro on the tumour cell lines used to establish the xenografts ($r^2=0.22$). Other studies have similarly found no direct correlation between uptake of radiolabeled anti-EGFR antibodies and EGFR expression levels, measured ex vivo by Western blot or by in vitro saturation binding assays, in human cancer xenografts in immunocompromised mice (212, 218). Cai and co-workers, however, found a reasonable correlation ($r^2=0.80$) between the tumour uptake of $^{64}$Cu-labeled cetuximab ($^{64}$Cu-cetuximab) and EGFR expression density of tumour tissue measured by ex vivo Western blot analysis. In my study, a non-linear relationship between tumour uptake of $^{111}$In-DTPA-trastuzumab and HER2 expression level was only observed after tumour uptake was corrected for non-specific IgG uptake and circulating radioactivity ($r^2=0.90-0.99$ and $r^2=0.87-0.95$). This non-linear relationship suggests that precise assessment of HER2 expression levels (receptors per cell) in patients with high HER2 expression may be difficult to achieve by non-invasive imaging. Molecular imaging, however, can easily distinguish between HER2 overexpression (i.e. IHC 2+ or 3+) and low HER2 expression (see sections 2.3.6-2.3.8), which is critical for stratifying patients who would be eligible for trastuzumab-based therapy. It may be likely that a single cutoff value (as used in FDG-PET) would be useful in defining HER2-positivity and predicting response to trastuzumab. Indeed, in Chapter 2 I demonstrated that the uncorrected tumour uptake of $^{111}$In-DTPA-trastuzumab was associated with therapeutic response in the limited panel of xenografts tested. In this study, a cutoff of 20 %ID/g would have accurately
predicted responding and non-responding tumours preclinically in these murine BC xenografts. In the case of a pre-defined cutoff value, tumour uptake will be dependent on the choice of imaging agent, target epitope, injected dose, radiolabel and imaging time post-injection, as all of these factors lead to significant variation in tumour accumulation of the radiopharmaceuticals (see section 1.8). Therefore standardization of imaging protocols will be extremely important for reliable, reproducible results.

5.2.3 Optimal imaging probe: size, affinity, valency, and specificity

For molecular imaging of BC an ideal imaging probe would localize specifically at high levels in tumour tissue and clear quickly from the blood and normal tissues. Additionally, the ideal probe would have 100% sensitivity and specificity for BC and specific accumulation of such an imaging probe would be indicative of a malignant lesion (100% PPV) and absence of specific accumulation would indicate the absence of disease (100% NPV). While no ideal imaging agent currently exists, significant improvements can be made by tailoring the size, affinity, valency and target epitope of different probes to attempt to address these optimal characteristics.

High tumour-to-blood (T/B) and tumour-to-normal tissue (T/N) ratios are essential for visualizing radiopharmaceutical uptake in lesions. As discussed in section 1.8, low molecular weight (MW) imaging probes typically have faster blood clearance and therefore higher T/B ratios at earlier time points. For imaging agents with MW below 60 kDa, tumour uptake is governed to a greater extent by affinity and valency rather than by size since imaging agents of this size are all rapidly cleared by the kidneys (244). For imaging with MW above 60 kDa, however, the molecular weight greatly affects the rate of clearance and therefore tumour accumulation (241). There is a compromise between high tumour accumulation and rapid blood clearance with the aim of achieving better T/B ratios. Due to their long circulation time antibodies typically achieve the highest tumour accumulation compared to antibody fragments, engineered constructs and affibodies, since the longer circulation time provides more passes through a tumour and opportunities for antigen/receptor binding (243). This long circulation time, however, results in diminished T/B ratios, especially at early time points (243). As discussed in section 1.8, lower MW imaging agents such as scFv’s have rapid blood clearance and high T/B ratios at early time points, however these agents have very low tumour
accumulation (%ID/g) which is a disadvantage for their use as imaging agents (365). Greater tumour uptake can be achieved, however, by improving the affinity and avidity of these radiopharmaceutical probes (243). Divalent imaging probes such as diabodies have significantly greater tumour localization compared to monovalent probes (244). Larger fragments, such as minibodies have been designed to increase the molecular size of the probe to improve tumour localization compared to smaller constructs, while still achieving faster blood clearance than intact antibodies and F(ab')2 fragments (253). Despite improved blood clearance compared to IgG’s, these constructs achieve optimal T/B ratios only at 48 h p.i. and therefore do not provide the early time point advantage of small antibody constructs and affibodies (253). In fact, microSPECT/CT imaging with intact trastuzumab and pertuzumab IgGs (111In-DTPA-trastuzumab and 111In-DTPA-pertuzumab) as shown in Chapters 2 and 3, achieved higher tumour accumulation and a superior T/B ratio at 72 h p.i. than HER2 minibodies labeled with 131I and 111In at 12-24 h p.i. (time of maximum tumour uptake), despite their lower circulating blood radioactivity (252, 253). At 48 h p.i., however, the 111In-labeled minibody had a much improved T/B ratio compared to intact antibodies (253). Presently, affibodies can achieve the highest T/B ratios (~40-100), while maintaining reasonable tumour uptake (~6-12 %ID/g) at very early time points (1-4 h p.i.) following injection. Furthermore, these agents are developed from phage display libraries and can be selected for a variety of cancer targets as well as pharmaceutical properties (e.g. affinity). These agents are thus very promising for development as cancer imaging probes.

Additionally, the Fc portion of antibodies and engineered antibody constructs greatly affects the clearance and biological half-life of antibody-based imaging agents (243). Altered pharmacokinetics can be achieved through mutation of key binding residues responsible for the interaction between antibodies and the neonatal Fc receptor (FcRn), which protects circulating IgGs from catabolism and regulates serum half-life (253, 366). By interfering with their interaction with FcRn, IgGs are cleared more rapidly from circulation. An anti-HER2 engineered construct scFv-Fc (~105 kDa) which contains an intact Fc domain with mutations in 3 key epitopes involved in FcRn interactions shows similar pharmacokinetics to that of the smaller minibody (~80 kDa) (366). This strategy could also be applied the full IgGs described in Chapters 2 and 3 of this thesis (e.g. 111In-DTPA-trastuzumab or 111In-DTPA-pertuzumab) in order to improve their blood clearance and possibly result in improved T/B ratios, and diminished non-specific accumulation in tissues.
Selection of an appropriate target tumour-associated epitope/receptor is also important for achieving optimal tumour uptake and maximizing the information obtained from the imaging study. For selecting eligible patients for targeted anti-cancer therapies, an imaging agent which binds to the same epitope as the drug is useful because it provides information about the availability of the binding site. Furthermore, if the imaging probe is a radiolabeled analogue of the drug, it will also provide valuable information regarding drug delivery, as shown in Chapter 2 using $^{111}$In-DTPA-trastuzumab for the detection of HER2-positive human breast cancer xenografts in athymic mice. However, if the imaging agent is going to be used in repeat imaging studies during the course of therapy or as a means to monitor response to therapy, then an imaging agent which binds to a different epitope on the tumour-associated target protein would be superior, to avoid a reduction in tumour uptake of the radiopharmaceutical due to competition for binding to the target by the drug. This rationale was used in Chapter 3 in selecting $^{111}$In-DTPA-pertuzumab as an imaging probe for $\textit{in vivo}$ detection of trastuzumab-mediated HER2 downregulation in athymic mice bearing HER2-overexpressing human breast cancer xenografts.

Another consideration in selecting an optimal imaging agent to accurately quantify HER2 density is the rate of receptor internalization and fate of the imaging agent following internalization into tumour and normal cells. Rapidly internalized probes that are radioiodinated could underestimate the level of HER2 due to intracellular catabolism with subsequent release of iodotyrosine from the cells. Thus, the radiopharmaceuticals synthesized in Chapters 2 and 3 were designed using radiometals, which following internalization and proteolytic degradation are trapped intracellularly, often resulting in improved tumour retention compared to radioiodinated imaging probes. Radiometals which are trapped intracellularly following internalization may, however, overestimate HER2 density if the receptor is recycled back to the cell surface and able to bind additional probes. This assumes, however, that the imaging probe is not recycled to the cell surface along with the receptors. Austin and co-workers have proposed that trastuzumab and pertuzumab are recycled with the HER2 receptor back to the cell surface, therefore it is unlikely that $^{111}$In-DTPA-trastuzumab or $^{111}$In-DTPA-pertuzumab used to measured HER2 expression levels $\textit{in vivo}$ in athymic mice in Chapters 2 and 3 would result in overestimation of HER2 expression (112). The fate of smaller antibody constructs and
affibodies following internalization will be important to select optimal imaging time points. Wållberg and co-workers have investigated the cellular processing of synthetic affibody molecules ($^{111}$In-DOTA-Z$_{HER2:342}$-pep2) bound to HER2 in SKOV3, SKBr-3 and BT-474 cells and found that $^{111}$In-DOTA-Z$_{HER2:342}$-pep2 is internalized slowly, with 60-80% remaining on the cell membrane after 24 hours (367). Furthermore, Orlova and co-workers directly compared the cellular uptake and tumour-targeting abilities of $^{124}$I-labeled trastuzumab ($^{124}$I-PIB-trastuzumab) and an $^{124}$I-labeled affibody molecule ($^{124}$I-PIB-Z$_{HER2:342}$) and found that trastuzumab was internalized more quickly than the affibody (260). Conveniently, due to the rapid blood clearance of affibodies, high-contrast images can be acquired at early time points (1-6 h p.i.) following administration. Taken together, the slow internalization and early time-point imaging of affibodies may allow for quantitative imaging of HER2 before significant internalization takes place, thus improving the quantitative accuracy and/or reproducibility of non-invasive imaging by avoiding this confounding factor. Similarly, the radiolabeled antibodies used in Chapters 2 and 3 are unlikely to overestimate HER2 expression levels, since recycling receptors are likely to remain associated with the radiolabeled antibodies. These imaging agents may, however, underestimate the extent of HER2 downregulation if significant internalization and degradation of the receptors occurs between the time of radiopharmaceutical injection and imaging, due to the residualizing nature of $^{111}$In.

### 5.2.4 Monitoring response to trastuzumab using molecular imaging

In addition to assessing the tissue concentrations of target proteins, molecular imaging has the potential to predict therapeutic response in individual patients. Changes in the uptake of imaging agents during therapy could be indicative of changes in the target protein expression and/or cellular processes in response to therapy. To provide predictive value, changes in tumour uptake must correlate with clinical response. Therefore, focusing on the mechanisms of action of drugs may provide useful targets or interrogation pathways for such imaging agents. For example, HER2 downregulation has been proposed as one of the mechanisms of action of trastuzumab (see section 1.3.5). In Chapter 3, I have demonstrated, for the first time, that trastuzumab-mediated HER2 downregulation can be sensitively detected by microSPECT/CT in vivo in athymic mice bearing HER2-overexpressing MDA-MB-361 xenografts using $^{111}$In-DTPA-pertuzumab. In this study, a 50% decrease in the tumour uptake of $^{111}$In-DTPA-pertuzumab was measured and detected by imaging following only 3 days of trastuzumab (Herceptin®) therapy, indicating that molecular imaging can sensitively detect early mechanistic changes in response
to trastuzumab treatment. Furthermore, microSPECT/CT imaging using $^{111}$In-DTPA-pertuzumab following three weeks of trastuzumab treatment revealed a more dramatic 78% reduction in tumour uptake of $^{111}$In-DTPA-pertuzumab, due to a considerable reduction in the proportion of viable tumour cells in response to therapy. Tumour uptake was reduced to a level that was not significantly different than the uptake in tumours blocked with a 100-fold excess of unlabeled trastuzumab ($7.6 \pm 0.4$ vs. $7.8 \pm 0. \%\text{ID/g}$, respectively; $P=0.6$), indicating that tumour uptake following 3 weeks of trastuzumab therapy was largely non-specific. Correcting for non-specific accumulation, the specific tumour uptake of $^{111}$In-DTPA-pertuzumab was reduced by 100% following 3 weeks of trastuzumab therapy. These results suggest that imaging using $^{111}$In-DTPA-pertuzumab may be useful during trastuzumab therapy for detection of a therapeutic response. My results are comparable to those of Smith-Jones and co-workers who reported a 50% reduction (80% reduction when adjusted for non-specific tumour uptake) of $^{68}$Ga-labeled DOTA-conjugated Herceptin F(ab')$_2$ ($^{68}$Ga-DCHF) in HER2-overexpressing BT-474 tumours 24 hours following treatment with the Hsp90 inhibitor, 17-AAG (234).

As discussed in Chapter 3, $^{111}$In-DTPA-pertuzumab or radiolabeled affibodies are more suitable than $^{111}$In-trastuzumab IgG or trastuzumab fragments for monitoring therapeutic response because these imaging agents bind to epitopes of HER2 distinct from that recognized by trastuzumab, allowing these probes to quantify HER2 expression levels in the presence of the drug. This is advantageous for maximizing tumour detection sensitivity for patients undergoing trastuzumab therapy at the time of imaging. Furthermore, the unique binding epitope on HER2 provides distinction between receptor blocking and receptor downregulation (as shown in Chapter 3). Interestingly, however, the inability to image the target receptor expression due to receptor blocking by a therapeutic drug, as in the case of the estrogen receptor in tumours treated with tamoxifen (see section 1.9.3.1), may also provide valuable information for predicting response in individual patients. Blocking of the trastuzumab binding epitope on HER2 may demonstrate adequate delivery of the drug, which may correlate with response in patients. In this case, radiolabeled imaging probes utilizing the trastuzumab CDR would be the most appropriate for detecting response. Presently, no studies have evaluated HER2 blockade as a target process for molecular imaging to predict response to trastuzumab, however, a study by Perik and co-workers performed serial imaging using $^{111}$In-trastuzumab in 14 patients with
HER2-positive MBC and found that more lesions were visible on the first scan (within 24 hours of the first trastuzumab infusion) compared to the second scan (after 12 trastuzumab infusions), possibly due to blocking of the receptors by trastuzumab (223). In this study, the percent decrease in tumour uptake of $^{111}$In-trastuzumab and its relationship with therapeutic response was unfortunately not reported.

Imaging receptor occupation and downregulation could be extended to other molecularly targeted drugs, such as cetuximab (Erbitux, Imclone Systems, New York, NY). Cetuximab is a mAb against EGFR which competitively inhibits the binding of EGF and other ligands such as TGF-α, by sterically blocking ligand-dependent receptor activation and downstream signaling (368). Binding of the bivalent antibody also induces receptor internalization and degradation leading to receptor downregulation (368, 369). Cetuximab is approved by the FDA and Health Canada for the treatment of EGFR-expressing metastatic CRC and EGFR-expressing advanced squamous cell carcinoma of the head and neck (SCCHN), however, clinical studies in CRC failed to reveal an association between EGFR expression levels measured by IHC and response to the drug (368). To maximize patient benefit of cetuximab, new methods for selecting eligible patients, as well as methods for monitoring response early in the course of treatment are needed.

Aerts and co-workers recently radiolabeled cetuximab with $^{89}$Zr ($^{89}$Zr-cetuximab) and, similar to my results reported in Chapter 2, found a disparity between EGFR expression levels and tumour uptake of $^{89}$Zr-cetuximab in a panel of EGFR-expressing xenografts in immunocompromised mice, indicating that other physiological factors, such as inadequate vasculature, reduced capillary permeability and differences in the level of necrosis may cause discrepancies in drug delivery (218). The authors propose that differences in drug delivery are a possible explanation for the discrepancy between tumour EGFR levels and response to cetuximab and therefore molecular imaging using radiolabeled cetuximab may be useful for selecting patients who are most likely to respond to this drug (218). Other groups have similarly radiolabeled cetuximab for SPECT and PET imaging of EGFR and proposed that radiolabeled cetuximab could be useful for rationally selecting patients for cetuximab therapy as well as monitoring response during treatment (214-217). Interestingly, however, an elegant study by Fan and co-workers demonstrated that simply blocking the EGF/TGF-α binding site with cetuximab Fab was not sufficient to impair growth of A431 cells and that only bivalent cetuximab F(ab’)$_2$ or IgG could induce receptor downregulation which was required for inhibition of EGFR-mediated proliferation of the cells (369). This *in vitro* study suggests that monitoring receptor occupation using radiolabeled cetuximab would provide information regarding delivery of the drug to the
tumour, but could not provide evidence of the key mechanism of action, receptor downregulation, required for cetuximab-mediated growth inhibition, since the imaging probe could not distinguish between receptor blocking and receptor downregulation. Therefore, the use of an imaging agent that binds to an epitope distinct from that of cetuximab, could be useful for in vivo detection of EGFR downregulation in response to cetuximab therapy as was shown in this thesis for probing a similar mechanism for trastuzumab using 111In-DTPA-pertuzumab (Chapter 3). Such an imaging probe may provide early indication of treatment response to cetuximab, which currently has a modest response rate (10-13%) when administered as a single agent for treatment of advanced SCCHN or CRC (370, 371). Both receptor blocking and receptor downregulation could be probed as methods to monitor response of patients to cetuximab with the aim to find the most sensitive and accurate imaging biomarker.

Presently, it is not known whether imaging only one mechanism of action of a drug that has multifactorial mechanisms will be indicative of an overall response to therapy. One possible solution would be to image multiple mechanisms. In the case of trastuzumab, other proposed mechanisms include: promotion of an immune-mediated response (ADCC), inhibition of angiogenesis, inhibition of HER2-ECD cleavage, inhibition of PI3K pathway signaling and induction of cell cycle arrest through upregulation of p27kip1 (see section 1.3) (80). Proteins that are up- or down-regulated during treatment are possible targets for molecular imaging because they may result in quantifiable changes in tumour uptake compared to baseline (pretreatment) levels; as shown in Chapter 3 with the detection of trastuzumab-mediated HER2 downregulation using 111In-DTPA-pertuzumab. In the case of trastuzumab, upregulation of p27kip1 may be a useful target for molecular imaging of response. This protein, however, exists within the nucleus of cells, restricting its availability to circulating molecular imaging probes, which typically target cell surface proteins. Our group has previously explored novel strategies for overcoming such delivery barriers by promoting the cellular and nuclear uptake of imaging probes through conjugation with HIV-1 tat peptides which enable membrane translocation and nuclear uptake (372, 373). We have previously demonstrated that changes in upregulation of the nuclear protein p27kip1 following treatment with trastuzumab resulted in increased retention of 111In-labeled anti-p27kip1 antibodies conjugated with tat (111In-anti-p27kip1-tat) in MDA-MB-361 BC cells in vitro and in MDA-MB-361 xenografts in athymic mice (372). A disadvantage of this
approach, however, is that it is non-specific and results in uptake in all types of tissue, reducing target-to-background ratios. Inclusion of a tumour-specific targeting moiety could improve delivery to these target tissues and reduce uptake in normal tissues. Cornelissen et al have recently investigated the cellular and nuclear uptake of a bispecific immunoconjugate (IC) targeted against EGFR and p27kip1 using $^{111}$In-labeled EGF conjugated with an anti-p27kip1 antibody and a peptide harboring a nuclear localizing sequence (NLS) ($^{111}$In-EGF-anti-p27kip1-NLS) (374). In this study it was hypothesized that the bispecific IC would bind to EGFR and be internalized into BC cells, where the NLS would route the IC to the nucleus and the anti-p27kip1 antibody would bind to upregulated p27kip1 following treatment with trastuzumab, causing preferential cellular retention of the radioimmunoconjugates. Indeed, higher levels of p27kip1 correlated with greater retention of the IC in vitro, however, unexpectedly trastuzumab resulted in downregulation of p27kip1 in some BC cell lines (374).

Functional imaging can also be used to monitor changes in cellular processes, such as glucose utilization, during treatment. An advantage of FDG-PET is that it does not rely on a single mechanism of action of the therapeutic agent, which may not be representative of the overall response to drugs with multifactorial mechanisms such as trastuzumab. In the case of chemotherapy, studies of neo-adjuvant therapy have shown that the optimal time for FDG-PET may be after the second cycle of chemotherapy. In these studies, a ≥50% reduction in tumour uptake of $^{18}$F-FDG correlated with a response. The effect of molecularly targeted therapies on tumour uptake of $^{18}$F-FDG has not been well studied. Furthermore, the relationship between inhibition of molecular targets, such as HER2 by drugs such as trastuzumab, and tumour glucose utilization has not been defined. In Chapter 4, we have shown for the first time that changes in tumour uptake of $^{18}$F-FDG were evident in responding HER2-positive human tumour xenografts in athymic mice following 16 days of trastuzumab therapy, whereas there were no changes in the tumour uptake of $^{18}$F-FDG in non-responding xenografts. Interestingly, the reduction in tumour uptake of $^{18}$F-FDG also correlated with a reduction in viable tumour cells. In Chapter 3, I showed that changes in HER2 density were apparent following 3 days of trastuzumab therapy, however there was no reduction in tumour uptake of $^{18}$F-FDG at this early time point (Chapter 4). Following 16 days of trastuzumab therapy, however, IHC analysis of excised tumours indicated a significant reduction of viable tumour cells, which may explain the 50% reduction in tumour uptake of $^{18}$F-FDG. Taken together, this suggests that loss of HER2 does not result in an immediate reduction in tumour glucose utilization. These results are in agreement with a study by Smith-Jones and colleagues who demonstrated that a reduction in HER2 caused by 17-AAG
did not result in a change in tumour uptake of $^{18}$F-FDG in BT-474 human BC xenografts (358). In contrast, Su and co-workers demonstrated a 40% reduction in tumour uptake of $^{18}$F-FDG following only 2 days of treatment with 70 mg/kg/d of gefitinib, a small molecule TKI of EGFR, in NSCLC (H3255 and HCC4006) and epithelial carcinoma (A431) xenografts (375). Similarly, in a small clinical study (n=5) of NSCLC patients there was a significant reduction in tumour uptake ($SUV_{\text{MAX}}$) of $^{18}$F-FDG after only 2 days of treatment, with gefitinib however the change in $SUV_{\text{MAX}}$ was greater following 4 weeks of treatment. In this study, all 4 patients with PR or SD had a >50% reduction in $SUV_{\text{MAX}}$ following 4 weeks of treatment, while the patient with PD had a 2-fold increase in $SUV_{\text{MAX}}$ (278). These studies demonstrate that differences in the mechanisms of action of molecularly targeted agents may result in varying outcomes on tumour glucose utilization and optimal timing of FDG-PET. Large clinical studies will be required to validate the relationship between tumour response to trastuzumab and changes in tumour uptake of $^{18}$F-FDG, determine the optimal timing for imaging, as well as determine a cutoff value with predictive significance. Chapters 3 and 4 demonstrate that different imaging probes may provide different sensitivities in detecting early response to targeted therapies. Imaging agents that probe the mechanism of action rather than general processes, such as glucose utilization, may be the most sensitive to detect early molecular changes in response to targeted cancer treatment.

5.3 Future research

There are many directions for continued research arising from the work presented in this thesis. Firstly, in my studies I demonstrated successfully that $^{111}$In-DTPA-pertuzumab could sensitively detect changes in HER2 tissue concentrations in vivo in MDA-MB-361 xenografts in athymic mice. While I was able to achieve excellent tumour uptake (> 30 %ID/g) and T/B ratios (> 5:1), visualization of the tumours by imaging could only be achieved at 72 h p.i. due to the long circulation time of antibodies. For translation into a clinical setting, it may be desirable and most practical to reduce the time between radiopharmaceutical injection and imaging. Additionally, this would permit the use of radioisotopes with shorter half-lives and reduce the radiation absorbed dose to the patient. Smith-Jones and co-workers have demonstrated that high quality PET images could be obtained at 3 h p.i. using $^{68}$Ga-labeled DOTA-conjugated Herceptin F(\text{ab}’)$_2$ fragments ($^{68}$Ga-DCHF) to image HER2-overexpressing BC xenografts in athymic mice
Therefore, generation of DOTA-conjugated pertuzumab F(ab')\textsubscript{2} fragments could be similarly useful for reducing the time between injection and imaging with this agent. Furthermore, a DOTA chelator facilitates the use of PET isotopes, which have higher sensitivity for detection compared to those used for SPECT due to the collimation required (155). Additionally, numerous studies have demonstrated that commercially available affibodies, namely, Z\textsubscript{HER2:342}, can achieve quite good tumour uptake, especially for such small molecular size imaging probes, and excellent T/B ratios at much earlier time points (1-4 h p.i.) compared to intact antibodies. Therefore, future work may involve comparing the ability of radiolabeled Z\textsubscript{HER2:342} affibody and radiolabeled DOTA-pertuzumab F(ab')\textsubscript{2} to detect trastuzumab-mediated HER2 downregulation in MDA-MB-361 xenografts. Both imaging agents could also be radiolabeled with $^{111}$In and $^{68}$Ga for comparison of the sensitivity and quantitative accuracy of SPECT and PET.

Secondly, the relationship between trastuzumab-mediated HER2 downregulation and therapeutic response has not been defined. To investigate this relationship, it would be useful to quantify tumour uptake of either Z\textsubscript{HER2:342} or DOTA-pertuzumab F(ab')\textsubscript{2} in a panel of BC cell lines which overexpress HER2, including cell lines with known trastuzumab-resistance, such as MKN7 (376) and 231-TrR1 (377). Tumour uptake in treated and control (PBS) mice could be compared and tumour growth could be monitored to determine the relationship between changes in HER2 density tissue concentration and response to trastuzumab.

Thirdly, in Chapter 4 I demonstrated for the first time that trastuzumab therapy resulted in a 60% reduction in the tumour uptake of $^{18}$F-FDG in MDA-MB-361 xenografts which responded to trastuzumab therapy, but no change in the tumour uptake in MDA-MB-231 xenografts, which do not overexpress HER2 and do not respond to trastuzumab. For confirmation and further understanding of the relationship between HER2 downregulation, response to trastuzumab and tumour uptake of $^{18}$F-FDG, it will be necessary to expand these experiments to a larger panel of HER2 overexpressing, responsive and resistant BC cell lines. FDG-PET could be performed on the same panel of BC cell lines proposed above. This would facilitate comparison between uptake of $^{18}$F-FDG and Z\textsubscript{HER2:342} or DOTA-pertuzumab F(ab')\textsubscript{2} to further investigate the relationship between HER2-downregulation, as well as basal levels of HER2 and tumour glucose utilization.

Fourthly, the use of molecular imaging to monitor receptor downregulation could be applied to anti-EGFR therapy, e.g. cetuximab. Currently there is no effective way to select patients who are likely to respond to cetuximab therapy. This has resulted in a low rate of
response (~10-12%) when cetuximab was administered as a single agent. Therefore, a means to detect early response in patients would allow non-responding patients to benefit from alternative therapy and reduce the cost associated with failed treatments. It has been shown in vitro that receptor downregulation is required for the growth-inhibitory effects of cetuximab in A431 cells (369). Therefore detection of receptor downregulation may be useful for imaging therapeutic response. Future studies may include the production of a radiolabeled antibody F(ab')2 such as mAb 2E9 which binds to an epitope of EGFR distinct from that recognized by cetuximab. SPECT and/or PET imaging could be performed on a panel of CRC or NSCLC cell lines with varying EGFR expression. Tumour treatment studies could be correlated to imaging to determine if cetuximab downregulation is associated with therapeutic response.

Lastly, Cornelissen et al have demonstrated the potential of molecular imaging probes for detecting upregulation of intracellular targets following trastuzumab therapy. Therefore, another future direction of this research could be the development of a bispecific imaging agent for the detection of phosphorylated Akt (P-Akt), a downstream protein kinase activated by HER2 signaling. As discussed in section 1.3.4, inhibition of PI3K/Akt signaling is one of the proposed mechanisms of action of trastuzumab. In contrast, persistent PI3K/Akt signaling, mediated by ligand-dependent HER2:HER3 heterodimers and/or upregulation of other growth factor receptors such as IGF-1R has been associated with resistance to the drug (378-380). Therefore, imaging persistent P-Akt expression may be a useful marker for resistance to trastuzumab and other anti-HER2 therapies. Imaging intracellular P-Akt may potentially be achieved using a bispecific imaging agent consisting of an internalizing targeting moiety (e.g. EGF, trastuzumab Fab, pertuzumab Fab, or HRG) and an anti-P-Akt antibody Fab joined by a linker. This bispecific conjugate could be conjugated with DOTA for radiolabeling with appropriate radionuclides for SPECT or PET imaging. High retention of the imaging probe in tumours could be indicative of incomplete blocking of HER2:HER3 signaling or compensatory signaling by IGF-1R, provided that it could be shown that tumour retention was correlated with the levels of P-Akt. A possible limitation to this method is that the use of a residualizing radiometal such as 111In or 64Cu could complicate the interpretation of the images.
The field of molecular imaging has the potential to revolutionize personalized cancer therapy by tailoring the management of disease to individual patients. This thesis is a small contribution to the great progress being made in the field; however, many questions remain to be answered and the true potential of molecular imaging in clinical oncology has yet to be realized.
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