SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Integrating Metabolic, Neo-Angiogenic, and HER2-Targeted Imaging to Characterize Breast Cancer in Preclinical Models

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I. Introduction

Breast cancer is one of the most common and deadly cancers globally, with 2.3 million women diagnosed and over 660,000 deaths reported in 2022, accounting for 11.6% of all cancer cases and nearly 7% of cancer deaths worldwide. It is the most prevalent and fatal cancer among women and ranks second in incidence and fourth in mortality across both sexes. While early detection and improved treatments have reduced mortality in high-income countries, rates continue to rise in low-income regions and among certain racial groups. Despite a slower annual increase of about 1% since the widespread adoption of mammography, the global burden is expected to grow significantly, with projected annual cases reaching 3 million and deaths 1 million by 2040. These trends underscore the urgent need for better diagnostics, classification, and therapies to reduce breast cancer's global impact.

Breast cancer presents significant variability across age, race, and biological characteristics, reflecting its heterogeneity in incidence, progression, and prognosis. The risk increases with age, peaking at 60–69 years, while younger women, particularly those aged 40–49, often face worse outcomes. Racial disparities are also evident, with White and Asian Pacific Islander women more likely diagnosed at early stages, whereas Black women exhibit higher rates of aggressive, high-grade tumours. These differences highlight the need for molecular classification of breast cancer to guide diagnosis and treatment.

Molecular subtypes are defined by receptor status and Ki-67 levels. Luminal A, the most common subtype (>60%), is ER+/PR+ (PR >20%), HER2−, with low Ki-67 (<14%), and generally has a favourable prognosis. Luminal B (≥10%) is more proliferative, either ER+/HER2− with high Ki-67 (≥14%) or ER+/HER2+. HER2-positive breast cancer (<10%) lacks ER and PR but overexpresses HER2 receptor. Triple-negative breast cancer (TNBC) is defined by the absence of ER, PR, and HER2 expression, representing a highly aggressive and heterogeneous.

Risk factors differ among subtypes. Family history influences subtype-specific risk, with BRCA1 linked to TNBC and BRCA2 to Luminal B. Reproductive factors such as later menarche and extended breastfeeding reduce risk for various subtypes, while hormone replacement therapy and delayed menopause increase risk for Luminal types. Oral contraceptives are particularly associated with TNBC. Demographic patterns show Luminal A more common among Caucasians and older women, while TNBC is more prevalent in younger

women and individuals of African descent. High breast density also increases HER2-positive cancer risk.

Histological and imaging features vary by subtype. Luminal A tumours are typically low-grade, with tubular or cribriform patterns, while Luminal B tumours are often higher-grade with micropapillary features. TNBC exhibits diverse morphology with necrotic cores and basal-like characteristics. Radiologically, Luminal A tumours are stiffer with speculated margins, aiding early detection. TNBC and Luminal B may appear edematous, while HER2-positive tumours show increased vascularization.

Treatment strategies differ across subtypes. Luminal types respond well to hormone therapies. HER2-positive cancers are treated with targeted therapies such as trastuzumab and pertuzumab. TNBC relies on chemotherapy, with growing interest in immune checkpoint inhibitors. Prognosis also varies: Luminal subtypes have the best survival but a higher risk of bone metastasis; HER2-positive cancers, though historically associated with poor outcomes, have improved significantly with targeted therapies but still show a high incidence of brain and liver metastases. TNBC, despite lower metastasis rates, has the poorest survival and highest recurrence risk.

Breast cancer heterogeneity further complicates treatment. Intertumour heterogeneity refers to subtype differences between primary and metastatic sites, while intratumour heterogeneity indicates varying subpopulations within a single tumour, affecting treatment response. Temporal heterogeneity arises over time, especially after therapy, leading to receptor changes and therapy resistance. Receptor conversion post-treatment is frequent, especially with PR and HER2. Emerging subtypes like HER2-low and ER-low tumours require adjusted therapies to improve efficacy and minimize toxicity. Additionally, other biomarkers like HER3, EGFR, VEGF, androgen receptors, and PD-L1 influence treatment decisions, especially in aggressive subtypes like TNBC.

Therefore, breast cancer is a biologically complex disease requiring precise, subtype-specific diagnostics and therapies. A thorough and dynamic assessment of molecular and histological features is critical for optimizing outcomes and adapting treatment strategies over time.

Biomarkers are objective indicators of biological processes that play a vital role in detecting, classifying, and monitoring breast cancer. They help characterize tumour heterogeneity and guide personalized treatment. Three main types of biomarkers are used:

biofluid biomarkers (e.g., ctDNA, cell-free DNA) offer non-invasive, serial monitoring of tumour progression, though they require high sensitivity and cannot localize disease; tissue biomarkers, assessed via biopsies using IHC or FISH, remain the gold standard for diagnosing subtypes like HER2 amplification but are invasive and limited by sampling bias; and imaging biomarkers, including MRI, CT, and molecular imaging (SPECT, PET), provide non-invasive, real-time spatial information crucial for detecting metastases, assessing tumour morphology, and evaluating response to targeted therapies.

II. Literature review of PET Molecular Imaging in Breast Cancer: Metabolic, Microenvironmental, and HER2 Imaging for Tumour Stratification

Molecular imaging using radiotracers provides insights into tumour biology, aiding in breast cancer characterization and therapy planning. Among these, [^{18}F]FDG PET is widely used due to its ability to assess tumour metabolism. Higher FDG uptake is generally associated with hormone receptor-negative, HER2-positive, and high Ki-67-expressing tumours, which tend to be more aggressive. However, its specificity is limited, as FDG uptake does not reliably differentiate HER2-positive from TNBC and correlates more strongly with tumour grade, size, stage, and markers like p53 and EGFR than with HER2 status. It shows a moderate correlation with Ki-67 ($r = 0.44$), suggesting some utility in proliferation assessment but limited precision in subtype classification.

[^{18}F]FLT PET, targeting thymidine kinase 1, offers a more specific measure of cell proliferation. Studies show FLT has a stronger correlation with Ki-67 ($r = 0.54–0.7$), especially in breast cancer, compared to FDG. However, FLT is more frequently used to monitor chemotherapy response rather than subtype classification.

[^{68}Ga]Ga-FAPI, which targets fibroblast activation protein in cancer-associated fibroblasts, has shown promise with higher uptake in HER2-positive tumours. While not directly linked to subtype classification, it surpasses FDG in lesion detection, particularly in challenging anatomical regions, making it a promising tool for staging and monitoring.

Angiogenesis imaging targets elements of tumour neovascularization. VEGF-targeted tracers, such as [^{89}Zr]Zr-DFO-bevacizumab and [^{64}Cu]Cu-DOTA-VEGFDEE, have shown potential but face limitations due to false positives from physiological or pathological neovascularization in organs like the brain and bones. Other targets include aminopeptidase N (CD13), with [^{68}Ga]-NOTA-c(NGR) demonstrating high affinity in preclinical models, though its application in breast cancer remains limited.

Integrin αvβ3, overexpressed on tumour endothelium, is a key target in angiogenesis. RGD-based radiotracers, particularly dimeric forms like [^{18}F]Alfatide II and [^{68}Ga]Ga-RGD, have shown differential uptake across subtypes, higher in HER2-positive tumours and lower in TNBC. The FDG-to-RGD uptake ratio offers an additional tool to refine subtype differentiation, with the highest ratios in TNBC and lowest in luminal B.

In summary, while no single tracer currently enables precise breast cancer subtype classification, combined use of multiple molecular imaging biomarkers, especially those targeting metabolism, proliferation, and angiogenesis, holds promise for improving diagnosis, monitoring, and treatment stratification.

HER2 imaging is vital in breast cancer diagnosis and treatment, with recent advances in molecular imaging, especially PET, showing great promise. Despite many HER2-targeted PET tracers developed, only a few have reached human trials, and none are yet standard in clinical practice. This gap emphasizes the need for further development to effectively integrate these tracers into routine care.

One of the most studied tracers is trastuzumab labelled with ^{89}Zr ([^{89}Zr]Zr-DFO-trastuzumab). Its long half-life suits the slow kinetics of antibodies. Clinical protocols often use an initial "cold" dose of trastuzumab before the radiolabelled tracer to reduce background, with imaging done 4–5 days later. This tracer can detect HER2 expression in metastatic sites including liver, bone, lymph nodes, and brain (where blood-brain barrier disruption occurs). However, discrepancies with IHC results exist, especially in bone and liver lesions, partly due to the instability of the chelator DFO and free zirconium accumulation in bone. Antibody metabolism in liver and lymph nodes can cause false positives. Despite limitations, this tracer is useful for therapy monitoring and outcome prediction, as seen in the ZEPHIR trial where it improved predictive accuracy combined with FDG PET/CT. New chelators like HOPO and DFO* and site-specific conjugation techniques have enhanced tracer stability and tumour uptake while reducing off-target accumulation.

^{64}Cu labelled trastuzumab ([^{64}Cu]Cu-DOTA-trastuzumab) offers a shorter half-life and potentially better image resolution. Similar to the zirconium tracer, administering cold trastuzumab beforehand improves tumour-to-background contrast, but visualization is limited in patients on trastuzumab therapy. This tracer also detects brain metastases effectively. However, ^{64}Cu can transchelate with serum proteins, demanding more stable chelators like

NOTA and NODAGA, which reduce off-target uptake and organ doses while maintaining specificity.

Pertuzumab, which binds a different HER2 epitope, is an alternative, especially during trastuzumab therapy when binding sites may be saturated. [^{89}Zr]Zr-DFO-pertuzumab has demonstrated high specificity and can image HER2 heterogeneity, including brain metastases. It also helps monitor therapy response, with uptake changes reflecting HER2 expression during treatments like T-DM1 or HSP90 inhibitors. Site-specific pertuzumab tracers improve tumour-to-organ ratios, enhancing clinical applicability.

Trastuzumab emtansine (T-DM1), an antibody-drug conjugate for resistant cancers, has been labelled with [^{89}Zr]Zr-DFO for imaging that stratifies HER2 expression and predicts response. Preclinical data show high HER2 specificity and strong correlation between tracer uptake and tumour response.

Smaller antibody fragments lacking Fc regions have faster clearance and can improve imaging contrast. ^{68}Ga labelled F(ab') and F(ab')2 fragments retain HER2 affinity and show high tumour-to-organ ratios, though kidney uptake remains a challenge. ^{64}Cu labelled Fab fragments with optimized chelators like MANOTA show better stability and tumour contrast. Site-specific labelling further enhances imaging quality. However, their rapid clearance may limit tumour uptake, leading to strategies like PEGylation or PASylation to extend circulation time.

Nanobodies, small camelid-derived antibodies, are even smaller and ideal for short-lived isotopes like ^{18}F. Nanobodies 2Rs15d and 5F7 target different HER2 domains, demonstrating high specificity, good tumour contrast, and reduced kidney radiation. Clinical studies confirm their safety and ability to detect tumour heterogeneity, though kidney uptake remains a concern.

Affibody molecules, small protein scaffolds, have very high HER2 affinity. Early affibodies showed high liver uptake that complicated liver metastasis detection. Modifications with cysteine residues and hydrophilic linkers improved labelling stability and reduced hepatic uptake without losing affinity. Clinical studies with [^{68}Ga]Ga-ABY-025 show effective discrimination of HER2 status and tumour heterogeneity, though correlation with IHC varies. Newer affibodies incorporate albumin-binding domains to lower kidney uptake and are explored for both imaging and radionuclide therapy.

Other small protein scaffolds, like ADAPT6 and DARPins, show promise due to high affinity and rapid clearance. They achieve excellent tumour contrast but carry potential immunogenicity risks. Small synthetic peptides targeting HER2 offer low immunogenicity and easy modification. Peptides such as KCCYSL and LTVSPWY have shown high affinity and specificity in preclinical models, with rapid renal clearance suited for imaging.

In conclusion, HER2-targeted imaging agents range from full antibodies to small proteins and peptides, each with unique strengths and limitations related to pharmacokinetics, specificity, and dosimetry. Continued development aims to optimize these tracers to improve breast cancer detection, treatment planning, and monitoring, ultimately integrating them into clinical practice for better patient outcomes.

III. Aims of studies

1. Metabolic Activity & αvβ3 Expression in Aggressive Breast Cancer

Angiogenesis in cancer involves increased pro-angiogenic signalling and suppressed anti-angiogenic factors. Given the high energy demands of the tumour microenvironment, this study aimed to explore the link between angiogenesis and glucose metabolism.

We used [^{68}Ga]Ga-NODAGA-c(RGDfK)$_2$, a dimeric RGD peptide targeting integrin αvβ3, alongside [^{18}F]FDG for dual-tracer PET imaging. Their short half-lives ($T_{1/2}$ of 68 minutes and 110 minutes, respectively) enabled longitudinal imaging to monitor tumour progression. Tumour-to-muscle ratios were evaluated in correlation with tumour growth using two xenograft models: 4T1 (fast-growing, triple-negative murine breast cancer) and MDA-MB-HER2+ (slower-growing, HER2-positive human breast cancer). These models represent two of the most aggressive subtypes. In addition, tumour heterogeneity was assessed in the 4T1 model to evaluate spatial variation in tracer uptake.

2. Preclinical Evaluation of [^{52}Mn]Mn-Trastuzumab for HER2 Imaging and Biodistribution

Manganese-52 (^{52}Mn) is an emerging radiometal for antibody-based imaging due to its 5.6-day half-life and favourable positron emission profile (29%, with low β+ energy), providing high-resolution images comparable to ^{18}F. Unlike ^{89}Zr or ^{64}Cu, ^{52}Mn also has a production advantage via medical cyclotron from natural chromium. However, dosimetry must be managed carefully due to high-energy γ-photons.

Macrocyclic chelators are preferred for ^{52}Mn due to their resistance to transchelation. Among these, DOTA and its derivatives are particularly suitable, offering high thermodynamic stability (log K > 19) and compatibility with temperature-sensitive antibodies like trastuzumab.

In our first study, we used DOTAGA-anhydride, a DOTA derivative, as a bifunctional chelator due to its regioselective reactivity and ability to conjugate without protective groups or side reactions. The resulting [^{52}Mn]Mn-DOTAGA-trastuzumab was tested in xenograft models to compare uptake in HER2-positive and HER2-negative tumours, with melanoma models as negative controls. We also assessed tracer uptake between orthotopic (in situ) and ectopic (subcutaneous) HER2+ tumours to investigate the impact of tumour environment on biodistribution.

In the second study, we developed a novel Mn(II)-specific bifunctional chelator, BPPA, a bispyclen derivative with a picolinate arm, designed for high labelling efficiency and in vivo stability. After conjugation with trastuzumab, the complex was labelled with ^{52}Mn and evaluated in PET/MRI imaging studies using HER2-expressing xenograft models. Orthotopic and ectopic tumour sites were compared for uptake patterns. This new conjugate was benchmarked against the [^{52}Mn]Mn-DOTAGA-trastuzumab tracer to evaluate performance in terms of tumour targeting and pharmacokinetics.

IV. Materials and Methods

1. Research 1: Temporal Assessment of Metabolic Activity and αvβ3 Expression in Aggressive Breast Cancer Models

This study utilized [^{18}F]FDG and [^{68}Ga]Ga-NODAGA-c(RGDfK)$_2$ to assess glucose metabolism and αvβ3 integrin expression in aggressive breast cancer xenografts. Both radiotracers were prepared under GMP conditions. [^{68}Ga]Ga-NODAGA-c(RGDfK)$_2$ was prepared with radiolabelling of NODAGA-RGD carried out using cyclotron produced ^{68}Ga, followed by purification with solid-phase extraction. Radiochemical purity exceeded 95%.

Female CB17 SCID mice were inoculated into the inguinal mammillary fat pad with either 4T1 (fast-growing) or MDA-MB-HER2+ (slow-growing) cells (5×10^6 cells/mouse). Tumour development was monitored, and imaging started when tumours became palpable (day 7 for 4T1, day 20 for MDA-MB-HER2+). PET/MRI scans were performed under isoflurane anaesthesia using nanoScan® PET/MRI. [^{18}F]FDG scans followed 12-hour fasting; [^{18}F]FDG scans and [^{68}Ga]RGD scans required 80-minute incubation time.

Scans lasted 20 minutes, followed by MRI for anatomical reference. Image reconstruction used a 3D-OSEM algorithm. Volumes of interest (VOIs) were drawn over tumours and muscle, corrected for spillover from the bladder. Standardized Uptake Values (SUVs) were calculated and tumour-to-muscle ratios analysed with Muscle-Spacing Correction Method. Histological validation was performed post-mortem using H&E and immunohistochemistry for GLUT1 and αvβ3. Data were expressed as medians with interquartile ranges and visualized using GraphPad Prism.

2. Research 2: HER2 Expression in Different Cell Lines and Inoculation Sites Assessed by [^{52}Mn]Mn-DOTAGA(anhydride)-trastuzumab

The radiopharmaceutical [^{52}Mn]Mn-DOTAGA-trastuzumab was synthesized by conjugating DOTAGA to trastuzumab followed by ^{52}Mn labelling. Radiochemical purity exceeded 95%. For in vitro binding, MDA-MB-HER2+ and MDA-MB-468 cells were incubated with the radiotracer, and uptake measured via gamma counting at various time points.

In vivo experiments used CB17 SCID and C57BL/6 mice inoculated with HER2-positive (MDA-MB-HER2+), HER2-negative (MDA-MB-468), or melanoma (B16F10) cells. Xenografts were established in different sites (inguinal fat pad, subcutaneous scapula), and PET scans were performed at multiple time points post-injection (4–120 hours).

Mice received 3.5 ± 0.6 MBq of the tracer intravenously under isoflurane anaesthesia. PET/CT or PET/MRI imaging systems were used depending on the group. SUVmean values were calculated for tumours and organs. Imaging data were processed using Nucline and InterView™ FUSION software.

Post-imaging, tumours were resected for H&E and HER2 immunohistochemistry. Statistical comparisons were made using two-way ANOVA with Tukey's test (GraphPad Prism).

3. Research 3: Evaluation of [^{52}Mn]Mn-BPPA-trastuzumab for Highly Specific HER2 PET Imaging

This study developed and evaluated [^{52}Mn]Mn-BPPA-trastuzumab as a novel HER2-targeting radiopharmaceutical. The BPPA ligand was synthesized and characterized via NMR and mass spectrometry. DOTAGA and BPPA were conjugated to trastuzumab, purified by ultrafiltration, and radiolabelled with ^{52}Mn under optimized pH and temperature conditions. Radiochemical purity and serum stability were validated via TLC and UPLC-RA-MS.

Relaxometric, kinetic, and stability properties of the Mn-complex were assessed through potentiometric titrations, NMR, and relaxivity measurements under physiologic conditions. Transmetallation kinetics with Zn(II) were monitored by changes in T_2 relaxation times.

CB17 SCID and BALB/c mice were inoculated with 4T1 (HER2−) or MDA-MB-HER2+ cells both subcutaneously and orthotopically. Tumour growth was monitored for 1–3 weeks. Radiotracers including [^{52}Mn]$MnCl_2$, unconjugated tracers, and antibody conjugates were injected intravenously (2.5–2.6 MBq). PET/MRI imaging included 90-minute dynamic scans followed by static scans at intervals up to 10 days. PET images were reconstructed using MLEM with attenuation correction.

SUVs were computed from defined VOIs over tumours and organs. Mice were euthanized post-study, and tumour samples underwent H&E staining and HER2 immunohistochemistry. Data were analyzed using ANOVA.

V. Results

1. Research 1: Temporal Assessment of Metabolic Activity and αvβ3 Expression in Aggressive Breast Cancer Models

This study examined the heterogeneity of radiotracer uptake in aggressive 4T1 and slower-growing MDA-MB-HER2+ tumours using [^{18}F]FDG and [^{68}Ga]Ga-NODAGA-c(RGDfK)$_2$. In 4T1 tumours, tracer uptake varied significantly across regions and time points. On days 10–11, the SUVmean tumour-to-muscle ratio in avid regions reached 18.62 (14.99–22.25) for FDG and 13.98 (10.58–17.38) for RGD, while non-avid regions displayed substantially lower ratios: 7.06 (6.19–7.93) and 6.85 (6.74–6.97), respectively.

The uptake trends over time in avid and non-avid areas showed synchrony between FDG and RGD, indicating linked metabolic and integrin activity patterns. Whole tumour analysis confirmed this: in 4T1 tumours, FDG uptake increased from 7.22 (7.00–7.43) at baseline to 9.05 (9.03–9.06) by the third scan, while RGD rose from 9.05 (6.13–11.96) to 12.63 (10.48–14.79).

In MDA-MB-HER2+ tumours, FDG uptake remained low but stable (2.04 at week 1 and week 2, then 2.45 in week 3), while RGD uptake decreased slightly over time (from 8.70 to 5.35). Despite lower values overall, correlation between tracers was still observed, albeit weaker than in 4T1.

Tumour size differed markedly: on day 8, 4T1 tumours reached 656.24 mm^3 (387.81–924.66), compared to 28.84 mm^3 (27.97–39.98) for HER2+ tumours. FDG ratios were consistently higher in 4T1 tumours than in HER2+ (e.g., 9.05 vs. 2.04 in week 2). The RGD ratio also followed this trend, though less starkly (12.63 vs. 7.50).

A strong positive correlation was found between 4T1 tumour size and both FDG and RGD uptake (R^2 values not provided but graphically evident). This correlation was not present in MDA-MB-HER2+ tumours.

Histological evaluation confirmed biological differences. 4T1 tumours exhibited rich stromal content and strong GLUT1 staining, especially in stroma-rich zones, suggesting high metabolic activity. HER2+ tumours showed more necrosis and heterogeneous GLUT1 distribution. Both tumour types expressed αvβ3 integrin, although HER2+ samples had slightly weaker and more uniform staining patterns.

2. Research 2: HER2 Expression in Different Cell Lines and Inoculation Sites Assessed by [^{52}Mn]Mn-DOTAGA(anhydride)-trastuzumab

In vitro, MDA-MB-HER2+ cells showed significantly higher radiotracer uptake at all time points compared to MDA-MB-468 cells. Uptake in HER2+ cells rose from 544.8 ± 161.4 to 671.5 ± 16.4 cpm/10^6 cells between 30 and 180 minutes. In contrast, HER2− cells only reached 355.4 ± 81.8 cpm at 180 minutes.

In vivo PET imaging demonstrated highest [^{52}Mn]Mn-DOTAGA-trastuzumab activity in blood pool initially, with liver, spleen, kidney, and lung showing moderate and consistent uptake. Breast tumours in HER2+ mice exhibited high SUVmean values beginning at 24 hours post-injection: 1.67 ± 0.19 vs. blood pool 1.16 ± 0.18 ($p < 0.01$). Ectopic tumours showed lower values (SUVmean: 1.11 ± 0.22 at 48 h), although still exceeding liver and lung uptake (0.74 ± 0.09 and 0.60 ± 0.10, respectively).

Orthotopic tumours consistently displayed stronger uptake than ectopic counterparts. By day 3, SUVmean reached 1.55 ± 0.21 (orthotopic) vs. 0.97 ± 0.11 (ectopic) ($p < 0.0001$). Imaging showed early tumour-to-background contrast in orthotopic HER2+ tumours, while ectopic and HER2− tumours showed lower uptake, typically concentrated at the periphery.

It was evident that the orthotopic HER2-positive tumours consistently exhibited dominance throughout all time points, although an increase in the ratios of the MDA-MB-468 xenografts was observed in the later scans. Specifically, the tumour-to-background ratios of the

orthotopic HER2-positive tumours were significantly higher than those of the melanoma at all scan time points, and notably higher than those of the HER2-negative xenografts throughout the study duration (two-way ANOVA, $p < 0.05$).

Histopathology revealed necrosis in HER2+ and B16F10 tumours, and dense stroma in HER2− tumours. HER2 staining confirmed high expression in HER2+ tumours and negligible levels in HER2− and melanoma samples.

3. Research 3: Evaluation of [^{52}Mn]Mn-BPPA-trastuzumab for Highly Specific HER2 PET Imaging

This study assessed [^{52}Mn]Mn-BPPA-trastuzumab's radiolabelling, stability, biodistribution, and specificity. Labelling was efficient at pH 7 and room temperature, achieving 99.93 ± 0.07% RCP and 0.085 ± 0.033 MBq/µg molar activity, outperforming DOTAGA-trastuzumab (88.37 ± 8.37% RCP, 0.0200 ± 0.002 MBq/µg). However, serum stability over 10 days declined to 71% for BPPA-trastuzumab, while DOTAGA-trastuzumab maintained over 90%.

In healthy mice, unchelated [^{52}Mn]$MnCl_2$ accumulated in liver and pancreas, while [^{52}Mn]Mn-DOTAGA and BPPA cleared rapidly. DOTAGA was excreted mainly via urine, whereas BPPA showed some fecal clearance and retained kidney-pancreas uptake.

The biodistribution of [52Mn]Mn-BPPA-trastuzumab was different than that of [52Mn]Mn-DOTAGA(pSCN-Bn)-trastuzumab. On the first 48 h, liver uptake was significantly higher than kidney uptake thereafter, blood pool and liver showed much faster clearance rate than kidney, pancreas, and salivary glands

In HER2+ tumour-bearing mice, [^{52}Mn]Mn-BPPA-trastuzumab uptake exceeded that of DOTAGA from 48 hours onward. Breast tumour SUVmean reached 7.60 ± 1.45 at 240 h (vs. 2.15 ± 0.51 in HER2− tumours, $p \leq 0.01$), while back tumour peaked at 10.08 ± 2.18 (vs. 2.22 ± 0.47, $p \leq 0.001$). Tumour-to-liver and tumour-to-muscle ratios confirmed superior contrast, especially in the BPPA group.

PET imaging revealed early and sustained uptake in HER2+ tumours using BPPA tracer, with clearer differentiation from HER2− tumours than DOTAGA tracer. Comparative analysis showed that from 48 h onward, BPPA-trastuzumab provided significantly higher uptake in HER2+ tumours (ANOVA, $p \leq 0.001–0.0001$).

Immunohistochemistry confirmed HER2 positivity in MDA-MB-HER2+ tumours (HER2 IHC 2+), while 4T1 tumours were HER2 negative. Histology revealed necrosis in HER2+ tumours and abundant stroma in HER2− tumours, consistent with tracer accumulation patterns.

VI. Discussion

Advances in tumour imaging continue to refine our understanding of breast cancer heterogeneity, particularly the relationships between metabolism, angiogenesis, and receptor expression. This integrated approach allows for more precise diagnostics, treatment planning, and response monitoring. The present three-part study explored the spatiotemporal interplay of glucose metabolism and angiogenesis, assessed HER2 expression in various tumour contexts, and evaluated a novel chelator–antibody complex for improving HER2-targeted PET imaging. Each component contributes essential insight to the development of more effective molecular imaging strategies in breast cancer.

1. Temporal Assessment of Metabolic Activity and αvβ3 Expression in Aggressive Breast Cancer Models

The first study confirmed a positive correlation between [^{18}F]FDG and [^{68}Ga]RGD uptake in 4T1 and MDA-MB-HER2+ tumour xenografts. The correlation was stronger in fast-growing 4T1 tumours, particularly in avid regions, where SUVmean tumour-to-muscle ratios for both FDG and RGD showed higher ratios in the fast growing tumours. This suggests a synchronised biological interplay between tumour metabolism and neovascular activity.

Several mechanisms support this observation. Angiogenesis, driven by hypoxia-induced VEGF and related pathways, requires extensive energy input, largely supplied through glycolysis. GLUT1 and GLUT3 upregulation in both tumour and endothelial cells enhances FDG uptake in angiogenic zones. The Warburg effect further elevates glucose metabolism in hypoxic regions, often localized along with active neovascularisation. Cancer-associated fibroblasts, abundant in triple-negative tumours like 4T1, also contribute to both angiogenesis and glucose metabolism, compounding the observed uptake in PET imaging.

Consistent with prior clinical data showing FDG–RGD correlations ($r \approx 0.9$ in some cancers), our findings align with reports suggesting stronger relationships in FDG-avid cancers like TNBC. Correcting for muscle reference improves correlation visibility due to RGD's

generally lower uptake. In 4T1 tumours, both FDG and RGD ratios rose in trends with tumour volume, indicating functional links between angiogenesis, metabolism, and growth.

HER2-positive tumours also showed correlation, though weaker. This could reflect subtype-dependent differences in tumour vasculature and metabolism, reinforcing the importance of subtype-specific imaging biomarkers.

Interestingly, the FDG-to-RGD ratio was markedly higher in 4T1 tumours, consistent with the aggressive, glycolysis-heavy phenotype of TNBC. These tumours also had larger volumes, over 650 mm^3 compared to 29 mm^3 in HER2+ xenografts by day 8, and higher stromal content, confirmed histologically, which may contribute to the elevated FDG uptake. Conversely, HER2+ tumours showed greater necrosis, limiting tracer penetration and explaining lower FDG activity relative to size.

Although RGD uptake alone may not distinguish between tumour subtypes, it provides high specificity for angiogenesis. Given the spatial heterogeneity and strong correlation with tumour volume, RGD may be more reliable for delineating gross tumour volume, particularly in radiotherapy planning and monitoring anti-angiogenic therapies. The difference in FDG/RGD correlation between 4T1 and HER2+ models further supports this targeted role.

From a methodological standpoint, the Muscle-Spacing Correction Method developed in this study offers an elegant solution for correcting bladder-related spillover in PET imaging without in vivo interventions. This approach could be applied in other contexts, such as neuroimaging, to address partial volume effects near high-uptake regions.

However, the study's small sample size (n = 2 for 4T1) limits statistical analysis. Further work with larger cohorts and diverse subtypes is needed to validate these preliminary correlations.

2. HER2 Expression in Different Cell Lines and Inoculation Sites Assessed by [^{52}Mn]Mn-DOTAGA(anhydride)-trastuzumab

Our second study examined [^{52}Mn]Mn-DOTAGA(anhydride)-trastuzumab across HER2+, HER2−, and melanoma tumour models. PET imaging demonstrated high tracer uptake in orthotopic HER2+ breast tumours, surpassing ectopic HER2+ tumours and major organs. This suggests that orthotopic sites provide a favourable microenvironment for tracer accumulation, likely due to increased perfusion, vascular density, and reduced interstitial pressure.

Uptake in HER2− tumours (MDA-MB-468) rose over time, despite their lack of HER2 expression, pointing to substantial non-specific binding. This may result from the larger size and stromal composition of these tumours. Histological evaluation showed that HER2+ and melanoma xenografts developed necrosis, likely limiting uptake by reducing perfusion and binding site accessibility. In contrast, stromal-rich HER2− tumours retained more tracer over time due to decreased clearance.

The observed ovarian and lacrimal gland uptake, despite the known absence of cross-reactivity between trastuzumab and murine HER2, was likely due to non-specific uptake or local immunoglobulin activity, especially in immunodeficient SCID mice. This highlights the value of PET for detecting unexpected tracer accumulation, contributing to safety and dosimetry studies.

Interestingly, despite high in vitro uptake in HER2+ cells, in vivo imaging did not maintain strong specificity. The use of DOTAGA(anhydride) instead of parent DOTA may reduce immunoreactivity, as seen in prior [^{111}In] studies. This underscores the importance of validating chelator–antibody combinations for both in vitro and in vivo performance.

Limitations include small group sizes (n = 2 for HER2−) and imaging modality discrepancies (PET/CT vs. PET/MRI), which can affect SUV calculations due to different attenuation correction algorithms. Use of tumour-to-muscle ratios mitigated these effects.

In summary, [^{52}Mn]Mn-DOTAGA(anhydride)-trastuzumab provided reasonable tumour contrast and tracer stability but lacked optimal specificity. Factors such as inoculation site, necrosis, tumour size, and stroma influenced uptake patterns, complicating interpretation without more rigorous immunoreactivity assessment.

3. Evaluation of [^{52}Mn]Mn-BPPA-trastuzumab for Highly Specific HER2 PET Imaging

To address specificity challenges, our third study evaluated [^{52}Mn]Mn-BPPA-trastuzumab, a novel tracer incorporating a rigid bispyclen-derived chelator with high stability (log K_MnL = 16.14, pMn = 10.98) and favourable relaxometric properties. Radiolabelling was efficient under mild conditions (room temperature, pH 7), yielding RCP >99%. Molar activity surpassed that of DOTAGA-trastuzumab, enhancing binding site availability.

In vivo, [^{52}Mn]Mn-BPPA-trastuzumab demonstrated significantly higher tumour uptake in HER2+ tumours compared to HER2− models starting at 48 hours. At 240 h, these values exceeded DOTAGA-trastuzumab uptake by several-fold. Tumour-to-muscle and

tumour-to-liver ratios were also markedly higher in BPPA tracers at all time points, enabling earlier and clearer HER2 differentiation.

However, serum stability studies showed that RCP declined to ~71% by day 10, compared to >90% in DOTAGA-trastuzumab. The maleimide linker may be susceptible to transchelation or enzymatic degradation, leading to free Mn(II) release and uptake in the pancreas, salivary glands, and kidneys, organs previously noted for Mn(II) accumulation. Improved linker chemistry is being investigated to resolve this.

Biodistribution of free [^{52}Mn]$MnCl_2$, [^{52}Mn]Mn-DOTAGA, and [^{52}Mn]Mn-BPPA in healthy mice showed that DOTAGA cleared predominantly via urine, while BPPA exhibited some faecal clearance and retained higher uptake in Mn-accumulating tissues at later time points. This suggests partial in vivo dissociation of Mn(II) from BPPA when unconjugated, although conjugation to trastuzumab enhanced stability.

Despite these limitations, [^{52}Mn]Mn-BPPA-trastuzumab offered superior HER2 imaging performance. Notably, it provided clearer differentiation between HER2+ and HER2− tumours earlier than DOTAGA-based tracers, even in the presence of complicating tumour features like necrosis or stroma. For instance, although 4T1 tumours exhibited high non-specific uptake with DOTAGA, BPPA retained selectivity for HER2+ xenografts, overcoming these confounders.

One limitation was inconsistent uptake patterns between orthotopic and ectopic tumours in the BPPA group, possibly due to technical variation in injection site placement. Nevertheless, the overall tumour-to-background ratios strongly supported the utility of BPPA for HER2 imaging.

4. Conclusion and Future Perspectives

Our integrated PET imaging approach revealed strong functional relationships between tumour metabolism, angiogenesis, and HER2 expression across different breast cancer models. FDG and RGD, especially [^{68}Ga]Ga-NODAGA-c(RGDfK)$_2$, PET provided complementary insights into tumour heterogeneity and progression. HER2−targeted imaging using [^{52}Mn]Mn-labelled trastuzumab showed good tumour contrast, but specificity and in vivo stability varied by chelator.

Among the agents tested, [^{52}Mn]Mn-BPPA-trastuzumab showed the best balance of tumour uptake, specificity, and imaging contrast, with the potential for early and accurate

HER2 assessment. Optimizing linker stability and further refining chelator chemistry will be critical for clinical translation.

Future studies should include immunoreactivity assays, blocking studies, and dosimetry modelling to assess therapeutic potential. Efforts must also bridge translational gaps between rodent models and humans, accounting for differences in FcRn expression, antibody clearance, and HER2 physiology.

Ultimately, multi-tracer PET imaging, combining metabolic, angiogenic, and receptor-targeted approaches, offers a powerful tool for personalizing breast cancer diagnosis and therapy.

VII. Summary

Our FDG/RGD study demonstrated that in the 4T1 TNBC model, both [^{18}F]FDG and [68Ga]Ga-NODAGA-c(RGDfK)$_2$ imaging revealed highly heterogeneous tumour structures, with tracer uptake showing linear correlations with tumour growth. 4T1 tumours exhibited higher FDG/RGD ratios compared to MDA-MB-HER2+, reflecting distinct metabolic–angiogenic profiles in aggressive tumours.

Our HER2-targeted imaging study using [^{52}Mn]Mn-DOTAGA(anhydride)-trastuzumab achieved consistently higher TBRs in orthotopic HER2-positive tumours than in ectopic HER2-positive, HER2-negative, and melanoma models across all imaging time points.

Our chelator development study with the novel BPPA ligand significantly outperformed DOTAGA, with [^{52}Mn]Mn-BPPA-trastuzumab demonstrating superior TBRs and 3–4 fold higher HER2+ versus HER2− contrast by day 7. This extended imaging window allows earlier and more specific differentiation of HER2-positive tumours.

In conclusion, integrating metabolic, angiogenic, and receptor-targeted imaging provides a robust approach to assessing breast cancer heterogeneity, enabling more precise characterization and treatment optimization. The combination of [^{18}F]FDG, [68Ga]Ga-NODAGA-c(RGDfK)2, and [^{52}Mn]Mn-based trastuzumab, particularly with the BPPA chelator, shows promise in improving imaging accuracy. [^{18}F]FDG and [68Ga]Ga-NODAGA-c(RGDfK)2 tracers effectively assess tumour heterogeneity, with notable correlations observed between the tracers and tumour growth, particularly in fast-growing TNBC models. This highlights their potential for monitoring aggressive tumour subtypes.

[^{52}Mn]Mn-trastuzumab imaging demonstrates excellent tumour contrast and tracer stability with [^{52}Mn]Mn-DOTAGA-trastuzumab, though improvements in specificity are required. Factors such as inoculation site, tumour characteristics, and microenvironment significantly influence tracer uptake. Meanwhile, [^{52}Mn]Mn-BPPA-trastuzumab shows superior HER2 specificity and tumour contrast, enabling earlier differentiation of HER2-positive tumours. Despite these advancements, the stability of [^{52}Mn]Mn-BPPA-trastuzumab requires further optimization to enhance its clinical utility. Together, these findings demonstrate the potential of integrating advanced radiotracers to improve breast cancer imaging and therapeutic planning

VIII. Publications list

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Registry number: DEENK/411/2025.PL
Subject: PhD Publication List

Candidate: Minh Toan Ngo
Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology
MTMT ID: 10098862

List of publications related to the dissertation

1. **Ngo, M. T.**, Vágner, A., Nagy, G., Ország, G., Nagy, T., Szoboszlai, Z., Csikos, C., Váradi, B., Trencsényi, G., Tircsó, G., Garai, I.: HER2 expression in different cell lines at different inoculation sites assessed by [52Mn]Mn-DOTAGA(anhydride)-trastuzumab.
   Pathol. Oncol. Res. 31, 1-12, 2025.
   DOI: http://dx.doi.org/10.3389/pore.2025.1611999
   IF: 2.3 (2024)
2. **Ngo, M. T.**, Nagy, T., Szoboszlai, Z., Csikos, C., Dénes, N., Furka, A., Trencsényi, G., Garai, I.: The Relationship of Metabolic Activity and αvβ3 Receptor Expression in Aggressive Breast Cancer Subtypes Tumors: a Preliminary Report.
   In Vivo. 39 (1), 160-171, 2025.
   DOI: http://dx.doi.org/10.21873/invivo.13814
   IF: 1.8 (2024)
3. **Ngo, M. T.**, Vágner, A., Nagy, G., Ország, G., Nagy, T. M., Csikos, C., Váradi, B., Sajtos, G. Z., Kapus, I., Szoboszlai, Z., Szikra, D. P., Trencsényi, G., Tircsó, G., Garai, I.: [52 Mn]Mn-BPPA-Trastuzumab: A Promising HER2-Specific PET Radiotracer.
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4. **Ngo, M. T.**: Novel Molecular Classification of Breast Cancer with PET Imaging.
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List of other publications

5. **Ngo, M. T.**, Lê, Á. N., Đinh, D. P. H.: The Impact of Chemotherapy on Cardiovascular Mortality across Breast Cancer Subtypes.
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   Inorg. Chem. Front. 10 (16), 4734-4745, 2023.
   DOI: https://doi.org/10.1039/D3QI00356F
   IF: 6.1

Total IF of journals (all publications): 25,8
Total IF of journals (publications related to the dissertation): 13,3

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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