First-in-Human Molecular Imaging of HER2 Expression in Breast Cancer Metastases Using the $^{111}$In-ABY-025 Affibody Molecule

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Short title: Imaging HER2 in Breast Cancer Metastases

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ABSTRACT

The expression status of human epidermal growth factor receptor type 2 (HER2) predicts the response of HER2-targeted therapy in breast cancer. ABY-025 is a small re-engineered Affibody molecule targeting a unique epitope of the HER2-receptor, not occupied by current therapeutic agents. This study evaluated the distribution, safety, dosimetry and efficacy of $^{111}$In-ABY-025 for determining the HER2 status in metastatic breast cancer.

Patients and Methods

Seven patients with metastatic breast cancer and HER2-positive (n=5) or negative (n=2) primary tumors received an intravenous injection with approximately 100µg/140MBq $^{111}$In-ABY-025. Planar gamma camera imaging was performed after 30 min, followed by SPECT/CT after 4, 24 and 48 hours. Blood levels of radioactivity, antibodies, shed serum HER2 and toxicity markers were evaluated. Lesional HER2 status was verified by biopsies. The metastases were located by $^{18}$F-FDG PET/CT five days prior to $^{111}$In-ABY-025 imaging.

Results

Injection of $^{111}$In-ABY-025 yielded a mean effective dose of 0.15 mSv/MBq and was safe, well tolerated and without drug related adverse events. Fast blood clearance allowed high contrast HER2 images within 4-24 hours. No anti-ABY-025 antibodies were observed. Normalizing metastatic uptake at 24h to uptake at 4h, the ratio increased in HER2-positive metastases and decreased in negative (p<0.05) with no overlap and confirmed by biopsies. In one patient, with HER2-positive primary tumor, $^{111}$In-ABY-025 imaging correctly suggested HER2-negative status of the metastases. The highest normal-tissue uptake was in kidneys followed by liver and spleen.

Conclusion

$^{111}$In-ABY-025 appears safe for use in humans and is a promising non-invasive tool for discriminating HER2-status in metastatic breast cancer, regardless of ongoing HER2-targeted antibody treatment.

INTRODUCTION
Today, treatment of breast cancer is based on the biological profile of the individual tumor. The human epidermal growth factor receptor type 2 (HER2) status is crucial to predict the response of HER2-targeted therapy(1). Patients with breast cancer overexpressing HER2 have improved survival when treated with HER2-targeting agents like trastuzumab, pertuzumab, and trastuzumab emtansine(2-10).

The analysis of HER2 expression is usually based on the surgical specimen of the primary tumor or, in case of neo-adjuvant therapy or inoperable disease, on biopsies from the tumor(11). The pathological analysis includes immunohistochemistry (IHC) and in some cases in situ hybridization (FISH). Therapy for patients with disseminated disease is often based on histopathological classification of the primary tumor and not of the metastases. Disparities in HER2 expression of primary breast cancer and metastases have been reported. Meta-analysis of 26 studies including 2520 patients revealed discordance in HER2-expression between the primary tumor and local lymph node metastases in the range of 2.4-7.2% and discordance to distant metastases in the range of 6.9-18.6% and an absolute variation for all studies in the range of 0-40%(12). A recent symposium publication, including 2845 patients, reported absolute variations in the same range(13). Another recent study on 182 patients, with 28% discordance, indicated that patients with loss of HER2-expression in metastases had shorter overall survival than patients with unchanged expression(14).

The biopsy procedure can be inconvenient or even harmful for the patient, demanding lesions of suitable size and carries the risk of sampling errors. Heterogeneity of HER2-expression within lesions and also differences in expression between lesions in the same patient further limit the use of biopsies for correct diagnosis. Thus, improved methods for determining the HER2-status in patients with metastatic breast cancer are needed to optimize treatment regimes. One approach is molecular imaging using a radiolabeled tracer targeting HER2.

Molecular imaging allows whole body detection of aberrant gene expression (i.e. proteomic abnormalities). Radiolabeled trastuzumab has been clinically evaluated as a HER2-
specific molecular imaging agent (15, 16). We used an imaging molecule with about 23 times smaller molecular weight; i.e. Affibody molecules. Preclinical studies have shown promising results with these (17). Affibody molecules (Affibody AB, Sweden) are small, ~6.5 kDa, protein imaging agents based on a non-immunoglobulin scaffold. Target-specific Affibody molecules are selected from a library of several billion unique variants providing high affinity binders to a variety of targets such as HER2 and have shown good imaging properties in xenograft models (17-23). The HER2 binding Affibody molecule used in this study binds with picomolar affinity to the extracellular domain 3 of the receptor, i.e. to an epitope not overlapping with the epitopes for trastuzumab (domain 4) or pertuzumab (domain 2), thus permitting imaging during ongoing antibody therapy (20, 24, 25).

Recently, clinical data using the first generation HER2-binding Affibody molecule, ABY-002, demonstrated the feasibility of HER2-imaging in breast cancer patients with SPECT (\(^{111}\text{In}\)) and PET (\(^{68}\text{Ga}\))(26). However, high liver uptake prevented visualization of liver metastases. ABY-025, used in the present clinical study, is a second generation Affibody molecule with improved biochemical and biophysical characteristics, designed by protein engineering using an iterative approach of changing 11 amino acids (about 20% of the molecule) outside the HER2 binding region (18, 27).

In this first-in-human study with \(^{111}\text{In}\)-ABY-025 SPECT/CT we evaluate safety and tolerability and explore the uptake in tumor metastases and background uptake in normal organs. It was also of interest to study the ability of \(^{111}\text{In}\)-ABY-025 to discriminate between HER2-positive and negative metastases.
PATIENTS AND METHODS

Patients

Seven female patients (mean age 61.3 years, range 46-70) on treatment for recurrent metastatic breast cancer were enrolled into the study (Table 1). Five of the patients were diagnosed with HER2-positive primary tumors and two had HER2-negative tumors and served as controls.

Inclusion and exclusion criteria

Protocol criteria for inclusion and exclusion are detailed in Supplementary material. Briefly, patients with diagnosis of metastatic breast cancer and known HER2 classification of primary tumor (HER2-positive: score of 3+ using HercepTest or FISH-positive, score 2+ with HercepTest and FISH-positive; HER2-negative: score 0 or 1+ using HercepTest, or score 2+ but FISH-negative) were potential participants. On-going treatment was not an exclusion criteria.

Approvals

The Swedish Medical Products Agency, the regional ethics committee in Uppsala and the radiation protection ethics committee in Uppsala approved the study. Written informed consent was obtained from all participants. The study was registered as a clinical trial with the identifiers EudraCT 210-021078-12 and NCT01216033.

Patient characterization and safety assessment

In accordance with the study protocol all patients underwent physical examination at least 7 days before, immediately before and 7 days after injection of $^{111}$In-ABY-025. Standard clinical chemistry of blood and urine was investigated according to approved protocol. Possible adverse effects were investigated orally and with written patient questionnaires before and after $^{111}$In-ABY-025 injection (day 0) and 1, 7, 21 and 42 days later. Blood samples for determination of shed serum HER2 were taken immediately before injection, and the assays (ADIVA Centaur HER2/neu-Test, Siemens Healthcare Diagnostics) were thereafter performed at Laboratory Limbach, Heidelberg, Germany. Presence of anti-ABY-025 antibodies was measured in samples
taken before, 21 and 42 days after injections, using an ELISA assay developed by Affibody AB and performed at Clinical Chemistry and Pharmacology Laboratory at Uppsala University Hospital, Sweden.

\textbf{\textsuperscript{18}F-FDG PET/CT and other clinical imaging}

The metastatic status of patients was known prior to inclusion based on conventional imaging. \textsuperscript{18}F-FDG PET/CT imaging was performed five days prior to the \textsuperscript{111}In-ABY-025 administrations to identify size and location of viable metastases in all patients. Patients fasted 6h before FDG injection. A scan (GE Discovery VCT) from head to thighs was performed 3 h after intravenous injection of 5 MBq \textsuperscript{18}F-FDG. A low-dose CT (auto-mA 20-80) without contrast enhancement was used for attenuation correction and anatomical location. PET images were reconstructed using a clinical protocol supplied by the vendor and all relevant corrections for quantitative imaging were applied. The acquired data were evaluated using Hybrid Viewer (Hermes Medical, Sweden) and Advanced Workstation (GE Healthcare). For each lesion detected by PET/CT, the maximum standard uptake value (SUV) was noted. The volume of each tumor lesion was calculated by a thresholding technique including all voxels with equal or more than 42\% of maximum SUV. Additionally, MR, ultrasound or contrast enhanced CT were applied when needed for biopsies and further patient management.

\textbf{\textsuperscript{111}In-ABY-025 imaging}

\textsuperscript{111}In-ABY-025 was prepared essentially as described earlier(18). GMP grade ABY-025 was provided by Affibody AB in vials containing 100 µg. ABY-025 was labeled with \textsuperscript{111}In at the Department of Nuclear Medicine, Uppsala University Hospital. Patients were not required to fast before injection. \textsuperscript{111}In-ABY-025, about 100 µg, with a mean activity of 142.6 MBq (range 131-154), was injected intravenously. At 4, 24 and 48 hours post injection, whole body planar scans were performed followed by SPECT/CT (Infinia Hawkeye 4, GE Healthcare) over individually selected areas of special interest, defined by findings in an initial planar scan (anterior and posterior) after about 30 min, as well as \textsuperscript{18}F-FDG PET/CT. Low dose CT scans without contrast
enhancement were acquired for attenuation correction and anatomical correlation. SPECT data were reconstructed with CT-based attenuation correction into a 128x128 matrix using an iterative reconstruction algorithm.

**Blood samples and biopsies**

Blood samples were collected at 10 and 30 min, 2, 6, 24, 48 hours and 7 days after injection to determine blood clearance kinetics. Following SPECT/CT results, optional biopsies were taken from suitable and clinically relevant lesions. In two patients (patient 1 and 2) metastases were surgically removed after the study. Biopsies were analyzed by IHC (HercepTest™, DAKO) to verify the HER2 status.

**Statistical analysis**

Quotients between the 24/4 or 48/4 hours uptake of $^{111}$In-ABY-025 were calculated and 108 metastatic lesions larger than 1.5 mL, as measured by FDG-PET/CT, were included in further analysis. The significance in difference between 4 and 24 hours uptake values in HER2 positive metastases was analyzed using the non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA). The significance in difference between quotient values and SUV for HER2-positive and HER2-negative metastases was analyzed using the non-parametric Mann-Whitney U-test. A two-sided p-value less than 0.05 was considered significant.

**Supplementary material**

Information on protocol inclusion and exclusion criteria, patient medical history, blood kinetics determination, $^{111}$In-ABY-025 imaging of normal tissue uptake, dosimetry, the dual time-point analysis, metastatic SUV measurements and biopsy data (including IHC and evaluation criteria) are provided in Supplementary material.

**RESULTS**

**Safety assessment**
The administration of $^{111}$In-ABY-025 was well tolerated. No clinically significant changes in laboratory evaluations or in vital signs were recorded. No anti-ABY-025 specific antibodies could be detected in any of the patients 3 and 6 weeks after exposure (data not shown).

**Pharmacokinetics, biodistribution and dosimetry**

Blood kinetics of $^{111}$In-ABY-025 and uptake in kidney, liver and spleen are presented in Table 2 (and Table S1 in Supplementary material). Clearance of $^{111}$In from blood was biphasic with first half-life 2.9±0.5 hours and second half-life 27±5 hours. In normal organs, the highest uptake was observed in the kidney followed by liver and spleen. Uptake in salivary glands and bowels was also visualized. No correlation was found between any organ uptake and shed serum HER2 (8.8 – 56 µg/L; the upper limit of normal is 15 µg/L, Table 1). The normal organ receiving the highest radiation dose was the liver, followed by the kidneys and spleen at 0.068±0.025, 0.020±0.006, and 0.005±0.002 mSv/MBq, respectively. The effective radiation dose for the patients was 0.15±0.02 mSv/MBq (21 mSv per patient).

**Uptake in metastases**

In the HER2-positive patients, large metastases (>> 1cm) identified by $^{18}$F-FDG PET/CT could be visualized with $^{111}$In-ABY-025 in the first whole body planar scan approximately 30 minutes after injection. Most metastases could be detected with high quality on images taken 4, 24 or 48 hours after injection (Table 3). The mean uptake of $^{111}$In-ABY-025 in HER2-positive metastases was at least one order of magnitude higher than a calculated uptake for homogeneous distribution of radioactivity in the body at 24 hours after injection. The HER2 positive metastases were visualized in different locations and tissues, see examples for patients 1, 2, 3 and 7 in Figure 1. A brain metastasis of patient 2, not seen with $^{18}$F-FDG PET, was clearly visualized with $^{111}$In-ABY-025 and confirmed as HER2-positive by IHC after surgical removal. The high HER2-expression in an adrenal gland metastasis in patient 3 was detected despite close proximity to the kidney. Bone metastases were clearly visualized in patients 2, 3 and 7. Patient 7 allowed biopsy of one bone metastases and HER2-positivity was confirmed by IHC. Liver
metastases were visualized in patient 2 (Figure S1 in Supplemental Materials). Biopsies from all
four patients (1, 2, 3 and 7) taken from lesions defined with $^{111}$In-ABY-025 SPECT were HER2
stained and scored HER2 3+.

Patient 4 was included as a HER2-positive patient based on IHC score 3+ for the primary
tumor. However, $^{111}$In-ABY-025 SPECT showed low or no uptake in the FDG-defined lesions
and the HER2-negative status of biopsies from these lesions was confirmed by IHC (Figure 2
and Supplementary material). Patients 5 and 6 had HER2 negative primary tumors and the
uptake of $^{111}$In-ABY-025 in their metastases was of low contrast and HER2-negative status was
verified by IHC on biopsies from patient 5 (patient 6 refused biopsy).

**Discrimination between metastases with high and low HER2 expression**

The quantitative $^{111}$In-ABY-025 uptake in metastases classified as HER2-positive (patients
1, 2, 3, 7) and HER2-negative (patients 4, 5, 6) was different both at patient and lesion level. The
uptake in HER2-positive metastases increased from 4 to 24 hours while the uptake in negative
metastases generally was lower and decreased from 4 to 24 hours. HER2-positive and HER2-
negative lesions could be discriminated by calculating the decay-corrected 24/4 hours uptake
quotient. HER2-positive and HER2-negative metastases showed invariably a quotient >1 and <1,
respectively (Figure 3). The difference between the groups classified as HER2-positive and
HER2-negative was significant using a rank test (p<0.05) with no overlap between the groups.
The discriminatory capacity of this quotient was verified by IHC on biopsied lesions.

Re-evaluation of the original primary tumor tissue from patient 4 showed a heterogeneous
HER2 expression, varying from 0 to 3+ (IHC examples in Figure 2A, B and C) but the tumor
was scored 3+ since more than 10% of the cells were 3+. The analysis of $^{111}$In-ABY025 uptake
in the metastases indicated low or no HER2 expression (arrow in Figure 3), i.e. are HER2-
negative. IHC analysis of the biopsy samples from patient 4 after $^{111}$In-ABY-025 imaging
showed scores from 0 to 1+ (IHC examples in Figure 2F, G and H). Thus, the biopsy analyzes of
patient 4 metastases supported the use of 24/4 hour quotients for discrimination.
DISCUSSION

The results of this first-in-human exploratory study indicate that $^{111}$In-ABY-025 can be used as a whole-body oriented and non-invasive agent to discriminate between HER2-positive and HER2-negative metastases. A single intravenous injection was well tolerated and safe and gave an effective patient dose of approximately 21 mSv. No drug-related adverse events or anti-ABY-025 antibodies were observed.

The rapid clearance of $^{111}$In from blood and normal organs allowed HER2 imaging of large ($>>1$ cm) metastases 30 minutes after injection and gave images with good contrast after 4, 24 and 48 hours. The levels of shed serum-HER2 did not appear to impact upon normal organ uptake or blood kinetics.

The high uptake of $^{111}$In-ABY-025 in metastases from patients 1, 2, 3, and 7 provided excellent HER2 visualization throughout the body. IHC analysis of biopsies confirmed the overexpression of HER2. Thus, imaging with $^{111}$In-ABY-025 can identify HER2-positive metastases. However, large lesions could also be visualized in HER2-negative patients 5 and 6, although with weak signals (Table 2). This can be explained by the fact that tumors with HercepTest scores 0 and 1+ may have up to 15-25,000 and 80-110,000 HER2 receptors per cell, respectively (28). Thus, SPECT-based imaging appears sensitive enough to visualize even low HER2-expression. Preclinical studies have shown that discrimination between tumors with high and low levels of HER2-expression is possible, either by using Affibody molecules with low specific radioactivity (23) or utilizing the fast clearance of radioactivity from tumors with low HER2 expression (29). In the present study the decay corrected 24/4 hour uptake ratio was used to determine the HER2-status at both patient and lesion level. The average $^{111}$In-ABY-025 uptake increased significantly in all lesions from 4 to 24 hours and remained increased at 48 hours for the HER2-positive patients 1, 2, 3, and 7. In contrast, lesions from HER2-negative patients 5 and 6 showed decreased $^{111}$In-ABY-025 uptake from 4 to 24 hours. The 24/4 hour
uptake ratios were invariably >1 for HER2-positive and <1 for HER2-negative lesions. The validity of this approach was supported by data from patient 4 (primary tumor HercepTest score 3+) demonstrating low $^{111}$In-ABY-025 uptake in the metastases. Analysis of all lesions from this patient, showed 24/4 hours quotients <1, typical for low HER2 expression and IHC analysis of biopsies confirmed low HER2-expression with HercepTest scores 0 or 1+.

The 24/4h hour quotient method requires a two-day protocol. A single time point protocol using SPECT/CT appears to be feasible (see Supplementary material), but may optimally require PET technique since the sensitivity and absolute quantification is better than for SPECT. Preclinical studies have demonstrated that Affibody molecules can be labeled with positron emitters such as $^{68}$Ga and $^{18}$F with preserved HER2-targeting capacity(22, 30-32).

Interestingly, the use of $^{111}$In-ABY-025 allowed HER2 imaging of known liver metastases in patient 2. This is an improvement since liver metastases could not be visualized using the first generation anti-HER2 Affibody molecule, ABY-002 (Figure S1 in Supplemental Materials)(26).

ABY-025, used in the current study, has been obtained by protein engineering to increase hydrophilicity, thermal stability, production characteristics(27) and, as shown in animal experiments, lower liver uptake(18). The present study suggests that the changes engineered into ABY-025 provide clinical utility. The physiological liver uptake varied between the patients. Patient 2 had the lowest physiological liver uptake and fasted before administration of $^{111}$In-ABY-025 while patients eating before administration had higher physiological liver uptake.

Radiolabeled trastuzumab has been evaluated earlier for HER2-imaging (15, 16) and has reported optimal image quality 4-5 days after injection (15) to compare with 4-24 hours using $^{111}$In-ABY-025. The limited amount of reported clinical studies does not permit a detailed comparison of sensitivity and specificity of radiolabeled trastuzumab versus $^{111}$In-ABY-025 in the clinical setting. The unique binding epitope of ABY-025 different from epitopes of both trastuzumab and pertuzumab (24) allowed imaging during trastuzumab treatment.
Our findings indicate that imaging of breast cancer metastases with \(^{111}\)In-ABY-025 is feasible and might be valuable for selection of patients that may, or may not, benefit from HER2-targeted therapies, hence improving treatment utility and cost effectiveness.

ACKNOWLEDGEMENT

We thank the Swedish Cancer Society for financial support (contracts 110565 and 120415), the staff at Department of Nuclear Medicine, Uppsala University Hospital and research nurse Jessica Barrefjord, Department of Oncology, Radiology and Radiation Sciences, Uppsala University, Sweden, for administration and patient care.
Table 1: Patient characteristics before injection with $^{111}$In-ABY-025. Abbreviations: ER=estrogen receptor, PgR=progesterone receptor, S=surgery, R=radiotherapy, C=chemotherapy, E=endocrine therapy, T=trastuzumab, L=lapatinib.

*For more information on therapy history, see Supplemental Material.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>HER2 status in primary tumor (HercepTest)</th>
<th>Primary tumor ER/PgR</th>
<th>Pretreatment serum HER2 (µg/L)</th>
<th>Metastases locations detected with $^{18}$F-FDG PET/CT</th>
<th>Injected $^{111}$In-ABY-025 (MBq)</th>
<th>On trastuzumab during imaging</th>
<th>Therapy history*</th>
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<tbody>
<tr>
<td>1</td>
<td>69</td>
<td>3+/-</td>
<td>21</td>
<td>lymph node</td>
<td>131</td>
<td>Yes</td>
<td>S, R, C, T, L</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>3+/-</td>
<td>26</td>
<td>lymph node, liver, bone</td>
<td>135</td>
<td>Yes</td>
<td>S, R, C, E, T, L</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>46</td>
<td>3+/-</td>
<td>56</td>
<td>lymph node, bone, liver, adrenal</td>
<td>139</td>
<td>Yes</td>
<td>S, R, C, T, L</td>
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</tr>
<tr>
<td>4</td>
<td>70</td>
<td>3+/-</td>
<td>8.8</td>
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<td>Yes</td>
<td>S, R, C, E, T, L</td>
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<tr>
<td>5</td>
<td>66</td>
<td>1+/-</td>
<td>16</td>
<td>lymph node, bone</td>
<td>137</td>
<td>No</td>
<td>S, R, C</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>0/-</td>
<td>12</td>
<td>bone, liver, lung</td>
<td>139</td>
<td>No</td>
<td>S, R, C, E</td>
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<tr>
<td>7</td>
<td>57</td>
<td>3+/-</td>
<td>23</td>
<td>lymph node, bone</td>
<td>154</td>
<td>Yes</td>
<td>S, C, E, T, L</td>
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Table 2: Uptake of $^{111}$In evaluated from the SPECT images 4, 24 and 48 hours after injection of $^{111}$In-ABY-025 in tumor free areas of the organs with highest uptake (upper part of the table). Blood levels of $^{111}$In analyzed in blood samples taken 10, 30 min, 2, 6, 24, 48 hours and 7 days after injection of $^{111}$In-ABY-025 (lower part of the table). Mean values and standard deviations calculated from all patients (n=7). %ID/g = Percentage of injected amount of radioactivity per g tissue.

<table>
<thead>
<tr>
<th>Time for analysis</th>
<th>4 hours</th>
<th>24 hours</th>
<th>48 hours</th>
<th>Time for analysis</th>
<th>4 hours</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney (left) %ID/g</td>
<td>$0.084 \pm 0.031$</td>
<td>$0.103 \pm 0.030$</td>
<td>$0.085 \pm 0.027$</td>
<td>Liver %ID/g</td>
<td>$0.032 \pm 0.013$</td>
<td>$0.025 \pm 0.010$</td>
<td>$0.022 \pm 0.009$</td>
</tr>
<tr>
<td>Kidney (right) %ID/g</td>
<td>$0.090 \pm 0.034$</td>
<td>$0.111 \pm 0.035$</td>
<td>$0.093 \pm 0.032$</td>
<td>Spleen %ID/g</td>
<td>$0.009 \pm 0.005$</td>
<td>$0.007 \pm 0.005$</td>
<td>$0.006 \pm 0.004$</td>
</tr>
<tr>
<td>Blood levels</td>
<td>10 min</td>
<td>30 min</td>
<td>2 hours</td>
<td>6 hours</td>
<td>24 hours</td>
<td>48 hours</td>
<td>7 days</td>
</tr>
<tr>
<td>%ID/g</td>
<td>$9.75 \pm 3.90 \times 10^{-3}$</td>
<td>$6.12 \pm 2.15 \times 10^{-3}$</td>
<td>$3.46 \pm 1.25 \times 10^{-3}$</td>
<td>$2.03 \pm 0.70 \times 10^{-3}$</td>
<td>$0.63 \pm 0.17 \times 10^{-3}$</td>
<td>$0.32 \pm 0.06 \times 10^{-3}$</td>
<td>$0.10 \pm 0.01 \times 10^{-3}$</td>
</tr>
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</table>
Table 3: Metastases (n=249) analyzed for $^{111}$In-ABY-025 uptake using SPECT/CT at different times after injection. The analyzed metastases were detected with $^{18}$F-FDG PET/CT 5 days before the injection of $^{111}$In-ABY-025.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Number of metastases detected with $^{18}$F-FDG PET/CT</th>
<th>Number of metastases with detectable $^{111}$In-ABY-025 uptake at 4 hours</th>
<th>Number of metastases with detectable $^{111}$In-ABY-025 uptake at 24 hours</th>
<th>Number of metastases with detectable $^{111}$In-ABY-025 uptake at 48 hours</th>
<th>Number of biopsies</th>
<th>HER2 status of biopsies (IHC)</th>
<th>HER2 status of biopsies from imaging data using 24/4 hours quotients</th>
<th>HER2 status of primary tumor (see Table 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>2*</td>
<td>17</td>
<td>10</td>
<td>13</td>
<td>13</td>
<td>1</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>3*</td>
<td>12</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td>1</td>
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<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>7*</td>
<td>21</td>
<td>16</td>
<td>21</td>
<td>19</td>
<td>1</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>4**</td>
<td>71</td>
<td>3</td>
<td>12</td>
<td>13</td>
<td>7</td>
<td>negative</td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>5**</td>
<td>79</td>
<td>37</td>
<td>37</td>
<td>36</td>
<td>1</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
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<tr>
<td>6**</td>
<td>46</td>
<td>30</td>
<td>32</td>
<td>34</td>
<td>0</td>
<td>no biopsy available</td>
<td>negative</td>
<td>negative</td>
</tr>
</tbody>
</table>

* No $^{111}$In-ABY-025 uptake was visible on SPECT images of a few small lesions defined as metastases by $^{18}$F-FDG PET/CT in patients 1, 2, 3 and 7. Due to the sensitivity limit of SPECT it is not known whether these few were HER2-positive or HER2-negative.

** The $^{111}$In-ABY-025 uptake seen in $^{18}$F-FDG PET/CT defined metastases from patients 4, 5 and 6 gave low signals from large metastases and no signals from small metastases. The reason for $^{111}$In-ABY-025 SPECT signals in some large metastases although low HER2 expression in IHC is discussed in the text.
Figures

Fig 1. Examples of imaging of HER2-expression in breast cancer metastases using $^{111}$In-ABY-025 SPECT/CT. Rows (A), (B), (C), (D) and (E) corresponds to patients 1, 2, 3, 4 and 7, respectively. Left column shows the positive IHC staining of the primary tumors, second column the $^{18}$F-FDG-PET/CT scans, third column the $^{111}$In-ABY-025-SPECT/CT scans and the forth column shows IHC staining of the metastases. The blue circles indicate the sites where biopsies were taken: patient 1 - lymph node metastasis, patient 2 - brain metastasis, patient 3 - adrenal metastasis, patient 4 - bone metastasis and patient 7 - bone metastases. Note that the primary tumor from patient 4 showed IHC 3+ staining and the bone metastasis in a spinal process showed high uptake of $^{18}$F-FDG. However, there was low (nearly no) uptake of $^{111}$In-ABY-025 in the metastasis indicating HER2-negativity and this was confirmed by the IHC negative staining of the biopsy. Patients 5 and 6 were HER2-negative both in IHC analysis of the primary tumor, in the $^{111}$In-ABY-025 scans of metastases and in IHC of the metastasis (IHC only from patient 5, patient 6 refused biopsy). The bars in all IHC images correspond to 50 µm.
**Fig 2.** Patient 4 changed from HER2-positive primary tumor to HER2-negative metastases. Column (A) shows examples of variations in HER2 expression (IHC) in the primary tumor. More than 10% of the tumor cells in the primary tumor had strong circumferential HER2 staining of their entire cell membrane and therefore the patient was declared HER2-positive. (B) shows the $^{18}$F-FDG PET/CT scan 5 days prior to SPECT. In total, 71 metastases were detected (Table 3). (C) shows the HER2 scan 4 hours after injection of $^{111}$In-ABY-025. No HER2-expressing metastases could be detected in this scan but three metastases with low HER2-expression could be detected in SPECT/CT sections after 4 hours and some more after 24 hours (Table 3). However the 24/4 hour quotients indicated HER2 negative metastases in all these metastases. This was verified by IHC analysis of biopsies. Column (D) shows examples of IHC analyzes of three different metastases; top photo: thyroid metastasis that scored 0, middle photo: calcified thyroid metastasis that scored 0 and bottom: bone metastasis that scored 1+. The bars in the IHC-images correspond to 50 μm.
Fig 3. Plots of $^{111}$In-ABY-025 uptake ratios in metastatic lesions defined by $^{18}$F-FDG-PET/CT. In total, 108 lesions were included in the analysis. (A) Average $^{111}$In uptake at 24 and 48 hours calculated for each patient with HER2-positive metastases and normalized to the uptake at 4 hours after injection. The $^{111}$In uptake increased significantly in all lesions from 4 (filled circles) to 24 hours (filled squares) and remained increased at 48 hours (triangles) after injection (Kruskal Wallis ANOVA, $p = 0.01$). (B) Average $^{111}$In uptake at 24 hours normalized to the uptake at 4 hours for patients 1, 2, 3 and 7 with HER2-positive metastases (filled squares) and for patients 4, 5 and 6 with HER2-negative metastases (open squares). There was no overlap between the values for HER2-positive and HER2-negative metastases and the difference was significant (Mann-Whitney, $p < 0.05$). Mean values and standard error estimates are given. The arrow in B indicates the data for patient 4, i.e. the patient that had a HER2-positive primary tumor but HER2-negative metastases.
REFERENCES


7. Perez EA, Romond EH, Suman VJ, et al. Four-year follow-up of trastuzumab plus adjuvant chemotherapy for operable human epidermal growth factor receptor 2-positive breast cancer:


SUPPLEMENTARY MATERIAL

Inclusion criteria

Age over 18 years, diagnosis of metastatic breast cancer; availability of HER2 determination status from primary tumor (HER2-positive: score of 3+ using HercepTest or FISH-positive, score 2+ with HercepTest and FISH-positive; HER2-negative: score 0 or 1+ using HercepTest, or score 2+ but FISH-negative); volumetrically quantifiable lesions on $^{18}$F-FDG PET/CT with at least one lesion with diameter of more than 10 mm outside of the liver and kidneys; $^{18}$F-FDG PET performed within 21 days before administration of $^{111}$In-ABY-025; ECOG performance status of \( \leq 2 \); life expectancy of at least 12 weeks, hematological, renal and liver function test results within the following limits: white blood cell counts >$2 \times 10^9$ cells/L, hemoglobin >80 g/L, platelets >$50 \times 10^9$ cells/L, ALT, ALP, AST \( \leq 5 \) times Upper Limit of Normal, bilirubin \( \leq 2 \) times Upper Limit of Normal, serum creatinine: within Normal Limits; negative pregnancy test, capability to undergo diagnostic investigations within the study, written informed consent.

Exclusion criteria

Known hypersensitivity to ABY-025; known hypersensitivity to Dotarem®; second, non-breast malignancy; active current autoimmune disease or history of autoimmune disease, active infection or history of severe infections within 3 months of enrollment; known HIV positivity or chronically active hepatitis B or C; administration of other investigational medicinal products within 30 days of screening; pregnancy or breast-feeding.

Patient medical history

Patient 1 was a 69 years old postmenopausal Caucasian woman. Cancer in the right breast was diagnosed 15 years prior to this study. Four years later an ER- and PgR-negative tumor in the contra lateral breast was found and the patient received adjuvant chemotherapy after breast conserving surgery. Mastectomy was performed three years later because of recurrent disease in the left breast. At this time HercepTest had been introduced and the tumors were HER2-positive (3+). Two years later a 4 cm diameter lymph node metastasis was diagnosed in the right axillary region together with a lung metastasis. It was HER2-positive (3+), ER- and PgR-negative and treated with trastuzumab and aromatase inhibitors (AI) during 2.5 years. She then received capecitabine and lapatinib followed by combinations of trastuzumab and vinorelbine and paclitaxel and liposomal doxorubicin as well as radiotherapy 10 months before entering this trial. She had stable disease on trastuzumab monotherapy when entering this study.
Patient 2 was a 57 years old postmenopausal Caucasian woman operated in 2008 because of a HER2 positive (3+), ER-positive and PgR-negative cancer in the left breast with twenty regional axillary lymph node metastases. Further investigations showed liver, lymph node and bone metastases as well as pleura effusion and a remaining tumor in the surgical scar. After induction therapy with trastuzumab and taxanes followed by a five month period with lapatinib and capecitabine, she switched to AI and trastuzumab, which she had until the inclusion in this trail. On a CT-scan less than a month before the trial a parameningeal lesion in the visual cortex was described as either a metastasis or menigioma. Biopsy later confirmed this lesion as a HER2-positive (3+) breast cancer brain metastasis.

Patient 3 was a 46 year old Caucasian woman diagnosed with left sided breast cancer in 2004. The primary lesion was a HER2-positive (3+), ER- and PgR-negative ductal cancer and she received neoadjuvant chemotherapy. One year later a local relapse was diagnosed as well as mediastinal lymph node and bone metastases. She was treated with trastuzumab and docetaxel but after one and a half years she developed inflammatory HER2-positive (3+) cancer in the right breast. After surgery in 2007 she then received capecitabine and lapatinib for two years until metastases in the liver, pleura and a suspect adrenal gland metastases was diagnosed. She was then switched back to trastuzumab combined with vinorelbine followed by liposomal doxorubicin.

Patient 4 was a 70 years old postmenopausal Caucasian woman diagnosed with right-sided breast cancer 5 years before entering this study. The primary lesion was ER-positive and PgR-negative and initially diagnosed as HER2-positive (3+). She received adjuvant radiotherapy, chemotherapy, tamoxifen, AI and one year of adjuvant trastuzumab. Nine month before entering this study she was diagnosed with metastases in lymph nodes, lung, bone and brain. She received radiotherapy against the brain metastasis and lapatinib and capecitabine as systemic therapy followed by trastuzumab and vinorelbine, which was given when entering this study. The metastases did not respond to these systemic therapies.

Patient 5 was a 66 years old postmenopausal Caucasian woman diagnosed with bilateral breast cancer in 2008. The tumor was ER- and PgR-negative and HER2-negative (1+). She had primary lymph node and bone metastases and since 2009 lung and pleural metastases. She first received docetaxel and then capecitabine, anthracyclins and finally gemcitabine and carboplatin when entering this trial.

Patient 6 was a 65 year old postmenopausal Caucasian woman diagnosed with a right-sided breast cancer 26 years before entering this study. The primary tumor was ER positive, PgR negative and HER2-negative (0). At 2008 she was diagnosed with bone, liver, lung and pleural metastases. She received chemotherapy with only a short break for treatment with tamoxifen.
She had been treated with anthracyclines and capecitabine and was on paclitaxel when enrolled in this trial.

**Patient 7** was a 57 year old postmenopausal Caucasian woman diagnosed with left sided breast cancer 5 years before entering this study. She had eleven positive lymph nodes and the primary tumor was ER- and PgR-positive and HER2-positive (3+). After surgery she received adjuvant chemotherapy followed by trastuzumab and tamoxifen. Three years later she was diagnosed with bone metastases and treated with docetaxel and trastuzumab. Endocrine therapy was then used for ten months and followed by five lines of chemotherapy with concomitant trastuzumab or lapatinib for about 1.5 years. At the time of enrolment in the study she was treated with carboplatin and trastuzumab.

**Blood kinetics determination**

Blood samples were collected at 10 and 30 min, 2, 6, 24, 48 hours and 7 days after injection to determine blood clearance kinetics. The vials for the blood samples were weighted with a 0.1 mg accuracy scale (Mettler PJ300). The amount of radioactivity in the vial was then measured using an automatic well counter (Wizard 3 (1480) from PerkinElmer). Results (%ID/g) with mean values and standard deviations are given in Table 2 in the manuscript and individual patient values in Table S1 below.

**18F-FDG-PET/CT imaging**

The method for 18F-FDG-PET/CT scanning is described in the manuscript. These scans were additional to other clinical diagnostic procedures. Approximately 150 of 249 lesions identified with 18F-FDG-PET/CT were also detectable with 111In-ABY-025 and considered to be metastases. Lesions seen with 18F-FDG-PET/CT but not seen with 111In-ABY-025 are probably metastases with low or no HER2-expression. It is also possible that they were HER2-positive but so small that the lower resolution and sensitivity of 111In-gamma camera imaging technology prevented them from being detected. A few lesions identified with PET were classified as non-tumor, based on all radiological and clinical information, none of these were SPECT-positive.

**111In-ABY-025 imaging and normal tissue uptake**

The patients underwent an initial planar (anterior and posterior) whole body planar scan at 30 minutes after injection and further planar and SPECT/CT (Infinia Hawkeye 4, GE Healthcare)
scans at 4, 24 and 48 hours after injection of $^{111}$In-ABY-025. Planar images were corrected for attenuation using a transmission scan with a $^{57}$Co flood source. Regions of interest were drawn over metastases and whole-body in the planar images to obtain the whole organ activities. In the attenuation-corrected SPECT images, 4 ml volumes of interest were drawn over metastases, liver, spleen and kidneys, giving organ activity concentrations. These were, for the normal organs, multiplied with standardized normal organ weights to obtain the whole organ activities. Integrated activity for the normal organs was calculated by a single-exponential fit to the 24 and 48 hours data points. Integrated activity for remainder of the body was calculated as whole body minus defined source organs. Results (%ID/g) with mean values and standard deviations for kidneys, liver and spleen are given in Table 2 in the manuscript and the values for individual patients in Table S1 below.
<table>
<thead>
<tr>
<th>Blood [%ID/kg]</th>
<th>10 min</th>
<th>10,03</th>
<th>8,01</th>
<th>15,01</th>
<th>4,00</th>
<th>7,60</th>
<th>14,49</th>
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<tr>
<td>30 min</td>
<td>6,16</td>
<td>6,16</td>
<td>5,71</td>
<td>8,71</td>
<td>2,71</td>
<td>4,65</td>
<td>8,76</td>
</tr>
<tr>
<td>2 h</td>
<td>3,71</td>
<td>3,01</td>
<td>3,62</td>
<td>3,59</td>
<td>1,80</td>
<td>2,63</td>
<td>5,82</td>
</tr>
<tr>
<td>6 h</td>
<td>2,15</td>
<td>1,66</td>
<td>2,28</td>
<td>1,91</td>
<td>1,12</td>
<td>1,69</td>
<td>3,36</td>
</tr>
<tr>
<td>24 h</td>
<td>0,62</td>
<td>0,50</td>
<td>0,64</td>
<td>0,54</td>
<td>0,46</td>
<td>0,73</td>
<td>0,95</td>
</tr>
<tr>
<td>48 h</td>
<td>0,31</td>
<td>0,25</td>
<td>0,32</td>
<td>0,29</td>
<td>0,30</td>
<td>0,42</td>
<td>NA</td>
</tr>
<tr>
<td>7 d</td>
<td>0,11</td>
<td>0,08</td>
<td>0,10</td>
<td>0,09</td>
<td>0,10</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**TABLE S1.** Table with %ID/g values for kidneys, liver, spleen and blood for individual patients as a function of time after $^{111}$In-ABY-025 injections. All blood values should be multiplied with $10^{-3}$. The values were calculated by dividing the measured radioactivity concentration with the administered amount of radioactivity. The activity concentration was measured with the absolute calibrated gamma camera. NA = not analyzed.

Images related to the liver uptake are shown in the figure below. The liver uptake varied between the patients. In spite of these variations we could detect HER2 expression in liver metastases from patient 2. Patient 2 had the lowest liver uptake and did not eat before the scan at the day of analyzes. Patients who were eating normally had higher liver uptake. Animal experiments indicated that the normal tissue uptake of $^{111}$In-ABY-025 was generally low, except for the kidneys as expected for radio metal-labeled tracers (ref 20 in the article). The figure below shows liver uptake examples from the studies by Baum et al (ref 26 in the article), our study and the study by Perik et al (ref 16 in the article).
**FIGURE S1.** (A) Liver uptake (arrow) 4 hours after delivery of the previously used Affibody molecule $^{111}$In-ABY-002 studied with a whole body scan as described by Baum et al (ref 26) (with permission from the Journal of Nuclear Medicine). (B) Liver uptake (arrow) 4 hours after delivery of the new re-engineered Affibody molecule $^{111}$In-ABY-025 studied with a whole body scan of patient 2 in the present study. (C) Liver (red) and metastases (yellow) uptake 24 hours after injection of $^{111}$In-ABY-025 (SPECT) in patient 2 in the present study. (D) Same as in (C) but with the CT picture superimposed (SPECT/CT) and the metastasis marked with a red cross. (E) Liver (red) and a metastasis (yellow) shown with SPECT/CT 4 days after administration of $^{111}$In-trastuzumab as shown by Perik et al (ref 16) (with permission from the Journal of Clinical Oncology).

**Dosimetry**

Absorbed radiation doses were estimated using OLINDA/EXM 1.1 (http://olinda.vueinnovations.com/licensing/olinda). The radiation doses to the liver, kidneys and spleen were 0.068±0.025, 0.020±0.006, and 0.005±0.002 mSv/MBq, respectively. The dose-limiting organ was the kidney. Effective doses for each patient are shown in Table S2 and the
mean effective dose considering all patients was $0.15 \pm 0.02 \text{ mSv/MBq}$ giving an effective patient dose of approximately $21 \text{ mSv}$.

TABLE S2. Effective doses for the individual patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Mean</th>
<th>StdDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective dose (mSv/MBq)</td>
<td>0.14</td>
<td>0.12</td>
<td>0.13</td>
<td>0.15</td>
<td>0.15</td>
<td>0.18</td>
<td>0.17</td>
<td>0.15</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The dual time-point analysis

Analysis was performed to evaluate the lesion uptake pattern over time. Activity in the metastases was measured using small volumes of interest from SPECT images corrected for attenuation and decay at all three time points. Analyses were performed on all lesions detected by FDG-PET/CT and SPECT/CT with subanalyses performed on the subject level (average of all lesions) and for biopsy-verified lesions. The average lesion uptake continued to increase from 4 to 24 hours in HER2-positive cases and decreased in HER2-negative cases ($p<0.05$ for both). These findings imply that relative to the early deposition at 4 hours, uptake continues to increase (by 21-43%) in lesions that are HER2-positive, differentiating this group of subjects with no overlap from the HER2-negative cases in which lesion uptake decreases (3-10%) ($p<0.05$, Mann Whitney U-test). Thus, serial imaging using $^{111}$In-ABY-025 SPECT at 4 hours and 24 hours appears to provide a diagnostic opportunity for differentiating HER2-positive from HER2-negative patients.

Analyzed metastases and biopsies

The NCCN guidelines (http://www.nccn.org/patients/guidelines/breast/) and the recommendations of the Swedish Breast Cancer Group (national guideline for treatment of breast cancer, written in Swedish) were considered. Decision about when a biopsy should be taken was decided by the responsible physician with patient consent. The Swedish Medical Products Agency, the regional ethics committee and the radiation protection ethics committee approved this procedure.

Detected lesions were considered to be metastases by two specialists in nuclear medicine. In total 255 lesions were detected and volume estimated with $^{18}$F-FDG PET. Analysis of each
patient includes comparison between metastases analyzed with $^{18}$F-FDG PET/CT and $^{111}$In-ABY-025 SPECT/CT and that was done for 249 of the detected metastases. The six lesions not included were not possible to evaluate with the SPECT technique since the analysis field was not exactly the same as for the PET investigations.

Biopsy samples were taken from the patients according to Table S3. The samples were fixed in 4% buffered formalin, processed and embedded in paraffin. Sections, cut 4-µm thick, were deparaffinized in xylene and hydrated through graded concentrations of ethanol to distilled water. Sections for routine analysis were stained with hematoxylin and eosin and sections for immunohistochemical, IHC, analysis were prepared according to the description by the Dako "HercepTest™ for automated link platforms, code SK001" (http://www.dako.com/se/ar39/p235367/prod_products.htm).

Bone metastases were, before embedding in paraffin, decalcified according to the protocol established in the department of pathology at Uppsala University Hospital. The solutions applied contained formic acid HCO$_2$H, sodium di-hydrogen citrate, C$_6$H$_7$NaO$_7$ and ammonium oxalate (NH$_4$)$_2$C$_2$O$_4$ and the procedure can be described in detail on request from the corresponding author.

The HercepTest scoring criterion was based on the recommendations in Dako HercepTest™ Interpretation Manual, Breast (http://www.dako.com/se/28630_herceptest_interpretation_manual-breast_ihc_row.pdf) where 0 corresponded to tumor cells completely negative, 1+ to faint perceptible staining of the tumor cell membranes, 2+ to moderate staining of the entire tumor cell membranes and 3+ was strong circumferential staining of the entire tumor cell membranes creating a fishnet pattern. The Canadian and the DAKO HercepTest guidelines (Mod Pathol 16:173-82, 2003) that require more than 10% of the tumor cells to be stained were applied. As positive controls we used in house positive control tissue sections as well as positive control sections supplied by DAKO. As negative HER2 controls we used normal tissues, which are expected not to express HER2 such as connective tissue and lymphocytes seen in the same sections as the metastases. We also used the surrounding capsule of lymph nodes as HER2 negative internal controls.
TABLE S3. Biopsies from metastases with corresponding HercepTest scores and 
\(^{111}\)In-ABY-025 SPECT Standard Uptake Values (SUV) at 24 hours after tracer injection.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Biopsies</th>
<th>HER2 score biopsy (IHC)</th>
<th>HER2 status primary tumor</th>
<th>SUV 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Surgical biopsy from axillary node metastasis</td>
<td>3+</td>
<td>Positive</td>
<td>8.5</td>
</tr>
<tr>
<td>2</td>
<td>Surgical biopsy from brain metastasis</td>
<td>3+</td>
<td>Positive</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>US-guided TruCut-biopsy from adrenal metastasis</td>
<td>3+</td>
<td>Positive</td>
<td>34.9**</td>
</tr>
<tr>
<td>4</td>
<td>US-guided TruCut-biopsy from thyroid metastasis</td>
<td>0</td>
<td>Positive</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>CT-guided biopsy from bone metastasis (L4 left pedicle)*</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CT-guided biopsy from bone metastasis (L4 spinal process)*</td>
<td>0</td>
<td>Positive</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CT-guided biopsy from bone metastasis (T5 left pedicle)</td>
<td>1+</td>
<td></td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>US-guided FNA (Fine needle aspiration) from supraclavicular lymph node metastasis</td>
<td>1+</td>
<td>Negative</td>
<td>2.7</td>
</tr>
<tr>
<td>6</td>
<td>No biopsy taken</td>
<td>-</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>CT-guided biopsy from sacral (S1) bone metastasis</td>
<td>3+</td>
<td>Positive</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Note: All bone biopsies were performed with use of 14G Bonepty bone biopsy system (Apriomed, Uppsala, Sweden). * No visible uptake compared to background. ** High value due to proximity to the kidney.

A possible single time-point assay

As a complement to the "dual time-point" analysis, described in the manuscript and above, we calculated a few examples for a possible future "single time-point" assay. We used data 24 hours after injection, visually regarded as the highest image quality. SPECT-based Standardized Uptake Values, SUV, were calculated as

\[
\text{SUV} = \frac{\text{ROI counts per mL} \times \text{calibration factor}}{\text{body weight in grams / injected dose}}
\]

A calibration factor for conversion of counts per mL to concentration was applied by repeated measurements of a 100 ml sphere containing a known amount of \(^{111}\)In and positioned inside a thorax phantom. The obtained 24 hours SUV for the biopsied metastases are shown in Table S3. Note that the SUV for patient 2 (brain metastasis scored 3+) was not very different from the SUV for patient 5 (lymph node metastasis scored 1+), i.e. 3.9 and 2.7, respectively. No calibration factor with a head phantom was available, which could have altered the SUV value. Note also that the 24 hours SUV for patient 3 (adrenal metastases) was extremely high, probably
due to signal spillover from the adjacent kidney. The adrenal metastasis could anyhow be easily distinguished from the kidney, as seen in Figure 1 in the manuscript.

Scanner resolution and scatter correction with SPECT/CT are less optimal than for PET/CT, which will affect the accuracy of measurements. In an attempt to reduce the impact of partial volume effects, we also performed a SPECT SUV-analysis for the largest metastases with diameter >3 cm, HER2-positive (n=9) and HER2-negative (n=8). One to 3 metastases were included per patient. The result is shown in Figure S2 and there was a significant difference in mean SUV (9.8±3.1 vs. 2.3±1.0, p<0.001). The data indicates that SUV at 24h reflects HER2 status. However, the "single time-point" assay is technically less applicable for routine clinical SPECT than the "dual time-point" assay (24/4 hour quotients). Furthermore, a quotient uptake approach is less affected by partial volume effects than are static concentration measurements. It is of course necessary to analyze and compare the different assays including more patients, which was beyond the scope of this formalized Phase I study. Most likely, a SUV-based "single time-point" assay probably can be used if a well calibrated PET scanning using a positron emitter is applied (e.g. $^{68}$Ga- or $^{18}$F- labeled ABY-025).
FIGURE S2. 24 h Standardized uptake values, SUV, of $^{111}$In-ABY-025 SPECT/CT in large metastases. To minimize the impact of partial volume effects only lesions larger than 3 cm were included (up to 3 lesions per patient). Red: HER2-positive and blue: HER2-negative. SUV was significantly higher in metastases from HER2-positive subjects (Mann-Whitney U test, $p=0.0006$) with no overlap between groups. The large adrenal metastasis (HER2-positive by biopsy) with SUV 34.9 was considered a measurement outlier due to signal spillover from adjacent kidney tissue and was removed in the analysis.