SUPPLEMENTAL 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 of 2+ with HercepTest and FISH-positive; HER2-negative: score of 0 or 1+ using HercepTest, or score of 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 the liver and kidneys; ^{18}F-FDG PET performed within 21 days before administration of ^{111}In-ABY-025; ECOG performance status of ≤2; life expectancy of at least 12 weeks; negative pregnancy test; capability to undergo diagnostic investigations within the study; written informed consent; hematological, renal, and liver function test results within the following limits: white blood cell counts $>2\times10^9$ cells/L, hemoglobin >80 g/L, platelets $>50\times10^9$ cells/L, ALT, ALP, AST ≤5 times upper limit of normal, bilirubin ≤2 times upper limit of normal, serum creatinine within normal limits.

Exclusion criteria

Known hypersensitivity to ABY-025; known hypersensitivity to Dotarem®; second, nonbreast 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-year-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 contralateral 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 that 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-year-old postmenopausal Caucasian woman who underwent surgery 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 took until inclusion in this trial. 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 meningioma. 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 suspected adrenal gland metastasis were diagnosed. She was then switched back to trastuzumab combined with vinorelbine followed by liposomal doxorubicin.

Patient 4 was a 70-year-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 months 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 were given when entering this study. The metastases did not respond to these systemic therapies.

Patient 5 was a 66-year-old postmenopausal Caucasian woman diagnosed with bilateral breast cancer in 2008. The tumors were 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, anthracyclines, and finally gemcitabine and carboplatin when entering this trial.

Patient 6 was a 65-year-old postmenopausal Caucasian woman diagnosed with right-sided breast cancer 26 years before entering this study. The primary tumor was ER-positive, PgR-negative, and HER2-negative (0). In 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 enrollment 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, and 48 hours; and 7 days after injection to determine blood clearance kinetics. The vials for the blood samples were weighed 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 article, and individual patient values are given in Supplemental Table 1.

^{18}F-FDG PET/CT imaging

The method for ^{18}F-FDG PET/CT scanning is described in the article. These scans were additional to other clinical diagnostic procedures. Approximately150 of 249 lesions identified with ^{18}F-FDG PET/CT were also detectable with ^{111}In-ABY-025 and considered to be metastases. Lesions seen with ^{18}F-FDG PET/CT but not seen with ^{111}In-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 ^{111}In-gamma camera imaging technology prevented them from being detected. A few lesions identified with PET were classified as nontumor based on all radiological and clinical information; none of these were SPECT-positive.

^{111}In-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

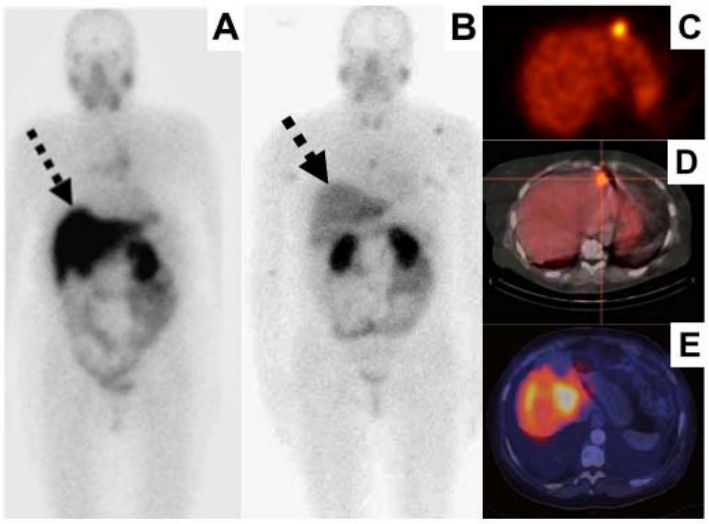
attenuation using a transmission scan with a ^{57}Co flood source. Regions of interest were drawn over metastases and the whole body in the planar images to obtain the whole-organ activities. In the attenuation-corrected SPECT images, 4-mL volumes of interest were drawn over metastases, liver, spleen, and kidneys, giving organ activity concentrations. These were, for the normal organs, multiplied by 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-hour data points. Integrated activity for the 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 article, and the values for individual patients are given in Supplemental Table 1.

SUPPLEMENTAL TABLE 1. 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 by the administered amount of radioactivity. The activity concentration was measured with the absolute calibrated gamma camera. NA = not analyzed.

Analysis	Time	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Right kidney	4 h	0.061	0.091	0.072	0.155	0.056	0.109	0.088
[%ID/g]	24 h	0.087	0.115	0.099	0.173	0.060	0.122	0.121
	48 h	0.070	0.091	0.085	0.145	0.047	0.117	0.092
Left kidney	4 h	0.055	0.090	0.075	0.143	0.048	0.097	0.083
[%/IDg]	24 h	0.075	0.121	0.099	0.151	0.058	0.109	0.107
	48 h	0.064	0.089	0.087	0.128	0.042	0.101	0.082
Liver	4 h	0.022	0.015	0.031	0.033	0.032	0.055	0.039
[%ID/g]	24 h	0.018	0.012	0.023	0.027	0.022	0.041	0.032
	48 h	0.016	0.010	0.019	0.024	0.021	0.037	0.027
Spleen	4 h	0.005	0.006	0.006	0.017	0.004	0.011	0.010
[%ID/g]	24 h	0.004	0.004	0.004	0.018	0.004	0.010	0.007
	48 h	0.003	0.004	0.004	0.014	0.004	0.009	0.007
Blood	10 min	9.07	10.03	8.01	15.01	4.00	7.60	14.49
[%ID/kg]	30 min	6.16	6.16	5.71	8.71	2.71	4.65	8.76
	2 h	3.71	3.01	3.62	3.59	1.80	2.63	5.82

6 h	2.15	1.66	2.28	1.91	1.12	1.69	3.36
24 h	0.62	0.50	0.64	0.54	0.46	0.73	0.95
48 h	0.31	0.25	0.32	0.29	0.30	0.42	NA
7 d	0.11	0.08	0.10	0.09	0.10	NA	NA

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 on the day of the analyses. 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 (*20*). The figure below shows liver uptake examples from the studies by Baum et al (*26*), our study, and the study by Perik et al (*16*).



SUPPLEMENTAL FIGURE 1. (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 (*26*). (B) Liver uptake (arrow) 4 hours after delivery of the new reengineered Affibody molecule ^{111}In-ABY-025 studied with a whole-body scan of patient 2 in the present study. (C) Liver (red) and metastasis (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 (*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 Supplemental Table 2, and the mean effective dose considering all patients was 0.15 ± 0.02 mSv/MBq, giving an effective patient dose of approximately 21 mSv.

SUPPLEMENTAL TABLE 2. Effective doses for the individual patients.

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Mean**	**SD**
Effective dose (mSv/MBq)	0.14	0.12	0.13	0.15	0.15	0.18	0.17	**0.15**	**0.02**

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 ^{18}F-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. The decision about when a biopsy should be performed was made 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 could not be evaluated 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 Supplemental Table 3. 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_2H, sodium dihydrogen citrate $C_6H_7NaO_7$, and ammonium oxalate $(NH_4)_2C_2O_4$, and the procedure can be described in detail by the corresponding author on request.

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+ to 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 that 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.

SUPPLEMENTAL TABLE 3. Biopsies from metastases with corresponding HercepTest scores and ^{111}In-ABY-025 SPECT standardized uptake values (SUV) at 24 hours after tracer injection.

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

Note: All bone biopsies were performed with use of 14G Bonopty 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

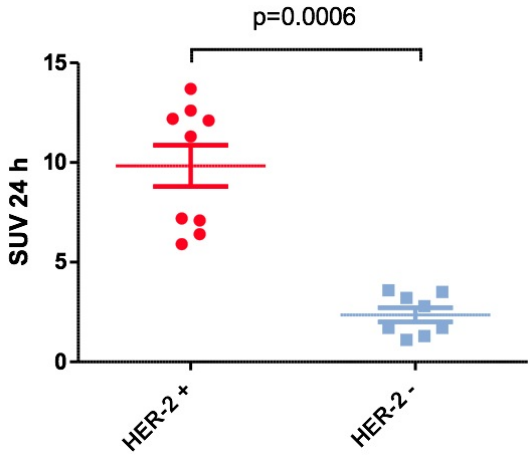
As a complement to the "dual-time-point" analysis, described in the article and above, we calculated a few examples for a possible future "single-time-point" assay. We used data 24 hours after injection, visually regarded as the highest image quality. SPECT-based standardized uptake values, SUV, were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

SUV = ROI counts per mL × calibration factor × 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-hour SUVs for the biopsied metastases are shown in Supplemental Table 3. 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. Note also that the 24-hour 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 article.

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 Supplemental Figure 2, and there was a significant difference in mean SUV (9.8±3.1 vs. 2.3±1.0, $P < 0.001$). The data indicate that SUV at 24 hours 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, an SUV-based "single-time-point" assay probably can be used if a well-calibrated PET scanner using a positron emitter is applied (e.g. ^{68}Ga- or ^{18}F-labeled ABY-025).



SUPPLEMENTAL FIGURE 2. 24-hour 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; 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.