Preparation of [^{nat}Ga/^{nat}In/^{nat}Lu]NeoBOMB1

NeoBOMB1 (600 μg) was incubated with a three-fold molar excess of the nitrate salt of ^{nat}Ga, ^{nat}In or ^{nat}Lu (Sigma-Aldrich) in acetate buffer (pH 4) at 90 °C for 30 min. The excess ^{nat}Ga, ^{nat}In or ^{nat}Lu was then scavenged by addition of EDTA. Metalated species, [^{nat}Ga/^{nat}In/^{nat}Lu]NeoBOMB1, were isolated from the reaction mixture by HPLC using a XBridge Prep Shield RP18 column (5 μm, 10 × 10 mm; Waters). The column was eluted at 1 mL/min flow rate applying a linear gradient consisting of 0.1% aqueous trifluoroacetic acid (TFA) (solvent A) and MeCN (solvent B): starting from 0% B to 20 % B in 5 min followed by a 1% increase of B over 60 min. The fractions containing [$^{nat}M^{3+}$]NeoBOMB1 were combined and lyophilized. The purity of the metalated DOTA-conjugates was assessed on an analytical reversed-phase column: Waters XBridge Shield RP18 (5 μm, 4.6 mm × 150 mm) cartridge column coupled to the respective 2-cm guard column. The column was eluted at 1 mL/min flow rate with a linear gradient of 0.1% aqueous TFA acid (solvent A) and MeCN (solvent B) starting from 25% B with a 0.5%/min increase of B over 60 min. t_R(NeoBOMB1) = 26.4 min, t_R([^{nat}Ga]NeoBOMB1) = 27.9 min, t_R([^{nat}In]NeoBOMB1) = 28.3 min, t_R([^{nat}Lu]NeoBOMB1) = 27.0 min.

Preparation of [^{125}I-Tyr^4]BBN

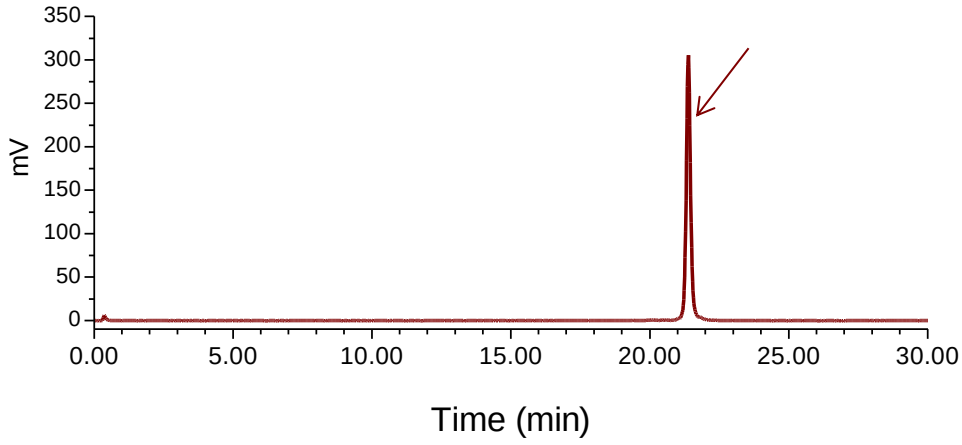
[Tyr^4]BBN (PSL GmbH) and ^{125}I (MDS Nordion) were used for the preparation of [^{125}I-Tyr^4]BBN. Radioiodination was performed by the chloramine-T methodology, as previously described (*S1, S2*). The forming sulfoxide (Met^{14}=O) was reduced by dithiothreitol and [^{125}I-Tyr^4]BBN was isolated in non-carrier added form by HPLC. Methionine was added to the purified radioligand solution to prevent re-oxidation of Met^{14} to the corresponding sulfoxide and the resulting stock solution in 0.1% BSA-PBS

was kept at −20 °C; aliquots thereof were used for competition binding assays (specific activity of 81.4 MBq/nmol). Samples were measured for radioactivity in an automatic well-type gamma counter (NaI(Tl)-crystal, Canberra Packard Auto-Gamma 5000 series instrument).

Preparation and Quality Control of [^{67}Ga/^{111}In/^{177}Lu]NeoBOMB1

Lyophilized NeoBOMB1 was dissolved in HPLC-grade H_2O (2 mg/mL) and 50 µL aliquots thereof were stored in Eppendorf Protein LoBind tubes at –20 °C.

Labeling with ^{67}Ga. Gallium (Ga-67) chloride in HC1 at an activity concentration of 1.2–2.4 GBq/mL was provided by IDB Holland B.V. [^{67}Ga]NeoBOMB1 was obtained at specific activities of 3.7–7.4 MBq ^{67}Ga/nmol NeoBOMB1. Briefly, in an Eppendorf Protein LoBind tube 3–15 nmol of NeoBOMB1 were mixed with 50–150 µL of 1 M sodium acetate buffer pH 4.0 and 5–15 µL of $^{67}GaCl_3$ (11–111 MBq). The mixture was incubated at 90 °C for 30 min. To scavenge non-peptide bound Ga-67 in the labeling solution EDTA was added to a final concentration of 1 mM. Reversed-phase HPLC was performed on a Waters Chromatograph (Waters) based on a 600E multisolvent delivery system coupled to a Gabi gamma-detector (Raytest, RSM Analytische Instrumente GmbH). Data processing and chromatography were controlled with the Empower Software (Waters). A Symmetry Shield RP18 cartridge column (5 µm, 3.9 mm × 20 mm) (Waters) was eluted at 1 mL/min flow rate with a linear gradient starting from 0% B and advancing to 60% B within 30 min (solvent A= 0.1% aqueous TFA and B= MeCN) (system 1). The radiochemical labeling yield (RCY) exceeded 98% and the radiochemical purity (RCP) was >99%; a representative radiochromatogram of [^{67}Ga]NeoBOMB1 is shown in Figure S1.



SUPPLEMENTAL FIGURE 1 Representative radioanalytical trace of [^{67}Ga]NeoBOMB1 labeling reaction mixture by HPLC (the radiopeptide t_R is indicated by the arrow).

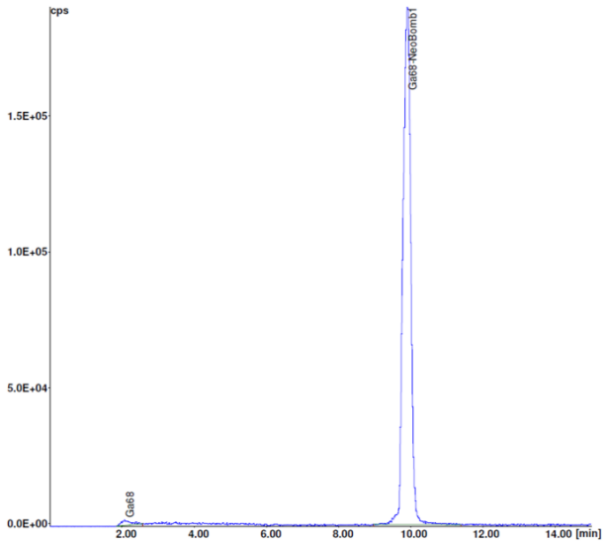
Labeling with ^{111}In. Indium (In-111) chloride in 50 mM HC1 was purchased from Mallinckrodt Medical B.V., at an activity concentration of 0.37–0.74 GBq/mL. [^{111}In]NeoBOMB1 was obtained at specific activities of 3.7–7.4 MBq ^{111}In/nmol NeoBOMB1. Labeling with ^{111}In was conducted in an Eppendorf Protein LoBind tube containing 1 M sodium acetate buffer pH 4.6. Freshly prepared sodium ascorbate buffer (10 mM) was added in the vial, followed by $^{111}InCl_3$ solution (37-74 MBq) and the peptide analog (10 nmol). The mixture was left to react at 80 °C for 20 min. Prior to performing quality control by HPLC, EDTA was added to a final concentration of 1 mM to the labeling reaction mixture to capture free $^{111}In^{3+}$. HPLC analysis was performed as described for [^{67}Ga]NeoBOMB1 (system 1). t_R ([^{111}In]NeoBOMB1) = 22.0 min

Labeling with ^{177}Lu. [^{177}Lu]Lutetium chloride was provided by IDB Holland B.V., in 50 mM HC1 at an activity concentration of 70-80 GBq/mL on calibration date. NeoBOMB1 was radiolabeled with ^{177}Lu at a specific activity of 37 MBq ^{177}Lu/nmol NeoBOMB1. Briefly, 10-20 nmol of NeoBOMB1 was mixed with 5-10 µL of 1.0 M

pH 4.6 sodium acetate buffer and 5-10 µL of $^{177}LuC1_3$ (350-800 MBq). Radiolysis was minimized by the addition of 5-10 µL of gentisic acid (80 mM) dissolved in 0.2 M sodium ascorbate. The reaction mixture was incubated at 85 °C for 15-20 min. For HPLC quality control (system 1) a 2 µL aliquot of the radiolabeling solution was quenched with 28 µL of an acetate buffered solution of DTPA (1 mM, pH 4.6). After a successful radiolabeling (more than 95 % NeoBOMB1-bound radioactivity) DTPA (10 mM, pH 4.6) was added to the radiolabeling solution to a final concentration of 1 mM. t_R ([^{177}Lu]NeoBOMB1) = 21.2 min

Preparation and Quality Control of [^{68}Ga]NeoBOMB1

All reagents used for the preparation of [^{68}Ga]NeoBOMB1 were purchased from commercial sources. Cartridges used for separation chemistry were bought from Varian and solutions were prepared with ultrapure water (Merck). All experiments were performed with a ^{68}Ge/^{68}Ga generator from Obninsk (Eckert & Ziegler Europe) or an IGG100 ^{68}Ge/^{68}Ga generator (Eckert & Ziegler Europe) eluate. The ^{68}Ge/^{68}Ga generator-eluate was purified and concentrated as previously described (*S3, S4*). An SCX-cartridge (Agilent Bond Elut SCX) was eluted with 500 µL of 5 M NaCl solution containing 12.5 µL of 5.5 M HCl directly into the reaction vial (about 1000 MBq ^{68}Ga). The vial was filled with 3 mL ultra-pure water, 350 µL (5 mg) ascorbic acid solution and 400 µL of 1 M sodium acetate pH 4.5. The end-pH of the reaction was in the range of 3.5 – 4.1. NeoNOMB1 (50 µg in 50 µL EtOH) was added and the reaction mixture was incubated at 95°C for 10 min. The radiolabeled product solution was neutralized by addition of phosphate buffer and passed through a sterile filter. Samples of the filtrate were taken for quality control (radio-ITLC and/or radio-HPLC), pH measurement, LAL pyrogenicity test, sterility test and storage.

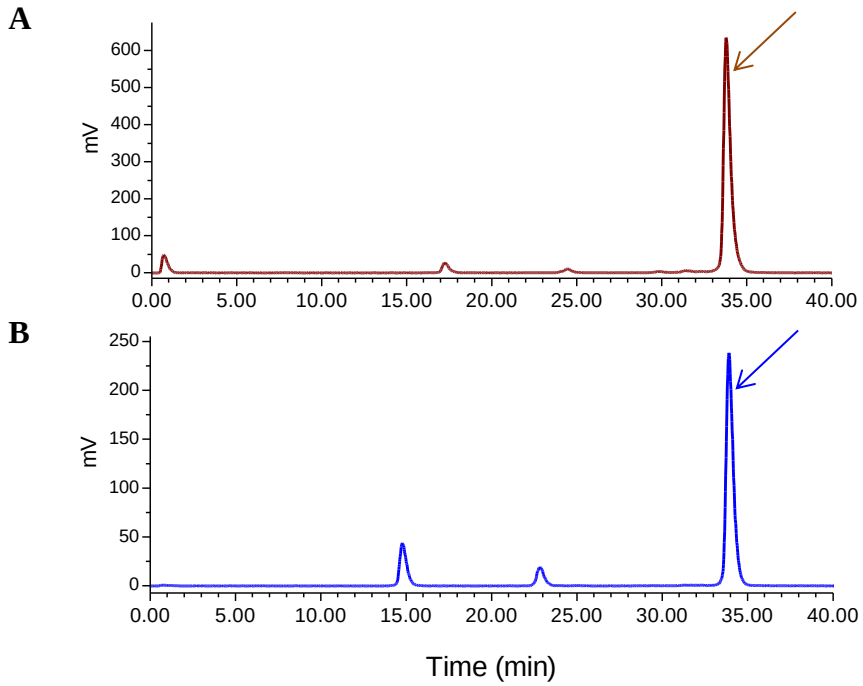


SUPPLEMENTAL FIGURE 2 Representative radioanalytical HPLC trace of [^{68}Ga]NeoBOMB1 labeling reaction mixture.

The quality control combined TLC and HPLC methods. ITLC was performed in two steps: a) ITLC-SG strips, solvent: sodium citrate buffer; R_f [^{68}Ga]NeoBOMB1 at origin, R_f free ^{68}Ga at the front; b) ITLC-SG strips, solvent: H_2O/MeCN 1:1 with 0.1% TFA; R_f ^{68}Ga-colloid at the origin, R_f ^{68}Ga-NeoBOMB1 ≈0.6, R_f free ^{68}Ga in between. The radio-scanner "Minigita" (Raytest) was used for radiodetection, typically revealing the absence of free ^{68}Ga or ^{68}Ga-colloid. For HPLC analyses the system applied included a Jasco PU-1580 pump of a quaternary gradient unit (Jasco LG-1580-04), a gamma detector (Biostep IsoScan LC) and a multi-wavelength detector (Jasco MD 1510). A RP-18 column (LiChroCART 250-4, LiChrospher 100, RP-18e; 5 µm, 250 mm × 4 mm) was eluted at 1.2 mL/min with the following gradient system: from 0–2 min 100% A, 2–15 min to 100% B, whereby A: 0.1% TFA in 5/95 MeCN/H_2O and B: 0.1% TFA in 95/5 MeCN/H_2O. The RCP, as determined by HPLC and expressed as the percentage of activity in the [^{68}Ga]NeoBOMB1 peak versus total activity, was >99%. As shown in Figure S2, the main peak for [^{68}Ga]NeoBOMB1 eluted at t_R 10.0 min

HPLC Analysis of Mouse Blood Samples

The stability of [^{67}Ga]NeoBOMB1 or [^{177}Lu]NeoBOMB1 in peripheral mouse blood was also tested at 30 min post injection (pi). The Symmetry Shield RP18 (5 µm, 3.9 mm × 20 mm) column was eluted at a flow rate of 1.0 mL/min with the following gradient: 100% A to 90% A in 10 min and from 90% A to 60% for the next 60 min; (A= 0.1% aqueous TFA (v/v) and B = MeCN).



SUPPLEMENTAL FIGURE 3 Representative radiochromatogram of HPLC analysis of mouse blood collected 30 min pi of (**A**) [^{67}Ga]NeoBOMB1 (>90% intact) and (**B**) [^{177}Lu]NeoBOMB1 (80% intact); the t_R of the parent radiopeptide is indicated by the arrow.

The position of intact radiopeptide was determined by coinjection with the [^{67}Ga]NeoBOMB1 or [^{177}Lu]NeoBOMB1 reference in the HPLC, respectively. The amount of intact [^{67}Ga]NeoBOMB1 at 30 min pi in mouse circulation exceeded 90% and of [^{177}Lu]NeoBOMB1 was ≈80% (Fig. S3).

[^{68}Ga]NeoBOMB1 PET/CT Imaging

Immediately prior to the ^{68}Ga NeoBOMB1 PET/CT study, the patients' weight and height were recorded. The patients were hydrated per os with at least 750 mL of bottled drinking water 1 h before of image acquisition. In order to increase renal washout and decrease radiation exposure to the urinary bladder, forced diuresis was performed with 20 mg of furosemide following i.v. injection of [^{68}Ga]NeoBOMB1.

All patients were examined on a dual-modality PET/CT tomograph (biograph duo; Siemens Medical Solutions, Erlangen, Germany). The CT component consists of a 2-row spiral CT system with a maximum continuous scan time of 100 s and a maximum rotation speed of 75 rpm. The PET component is based on a full-ring lutetium ortho silicate (LSO) PET system. Acquisition started 45-85 min pi of 60-339 (median 199.5) MBq [^{68}Ga]NeoBOMB1. All patients were required to drink 1.5 L of water-equivalent oral contrast solution (Gastrografin). Immediately before the PET/CT examination, patients were requested to empty their bladder. Patients were positioned supine with raised arms and head-first on the common patient handling system in accordance with standard CT practice.

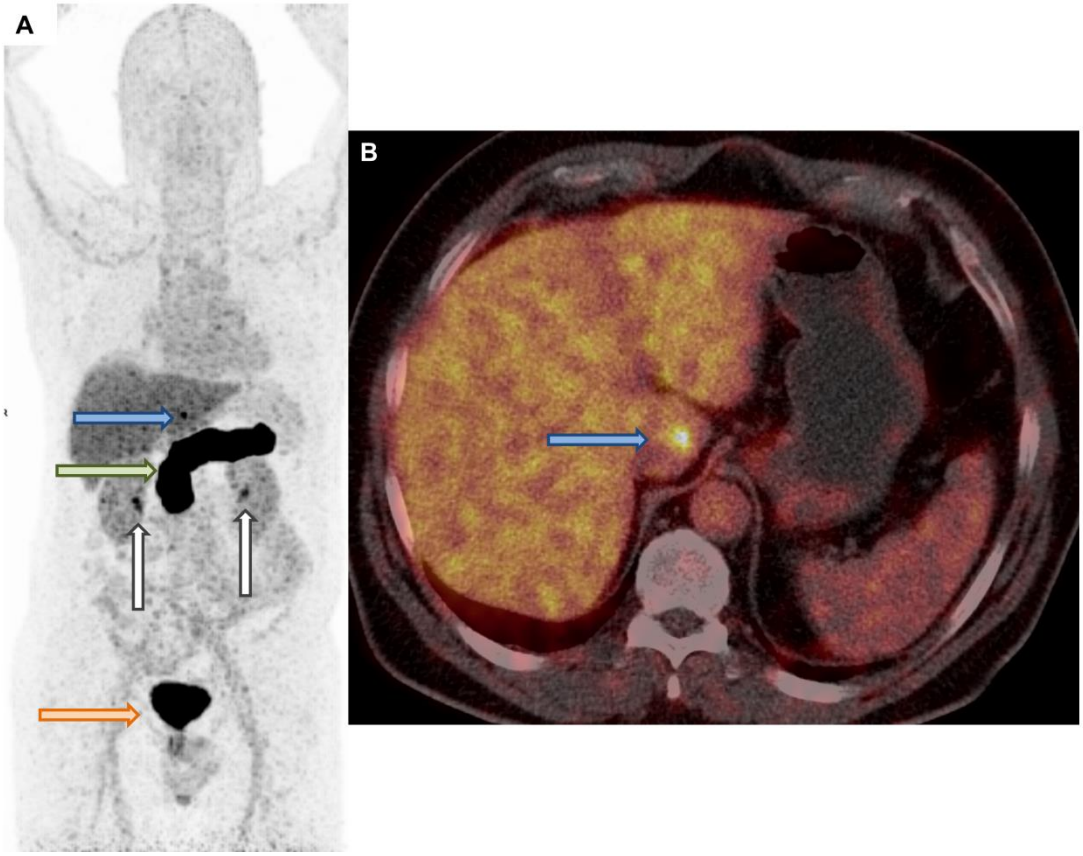
First, a topogram was acquired over 1,024 mm axially. Coaxial whole-body imaging ranges were defined on the topogram, covering an area from the skull to the mid-thighs (6-7 PET bed positions, or 90–110 cm, depending on the size of the patient). Intravenous contrast (100 mL) was administered by an automated injection pump, followed by CT scanning in the craniocaudal direction with a 30 s delay. CT was performed in spiral mode using a continuous acquisition at 130 kVp, 115 mAs, 4 mm collimation, 5 mm slice width, a table feed of 8 mm per rotation at 0.8-s rotation time, and 2.4 mm slice spacing. During the CT acquisition a limited breath hold protocol was followed, which required the patients to hold their breath in normal expiration. After completion of the

CT, patients were moved automatically to the PET towards the rear of the gantry, where 3D PET emission scanning subsequently started in the caudocranial direction. An emission scan time of 2-3 min per bed position was used for all patients, which resulted in a total emission scan time of no more than 24 min and a total PET/CT examination time of about 30 min (including patient positioning, CT and PET imaging).

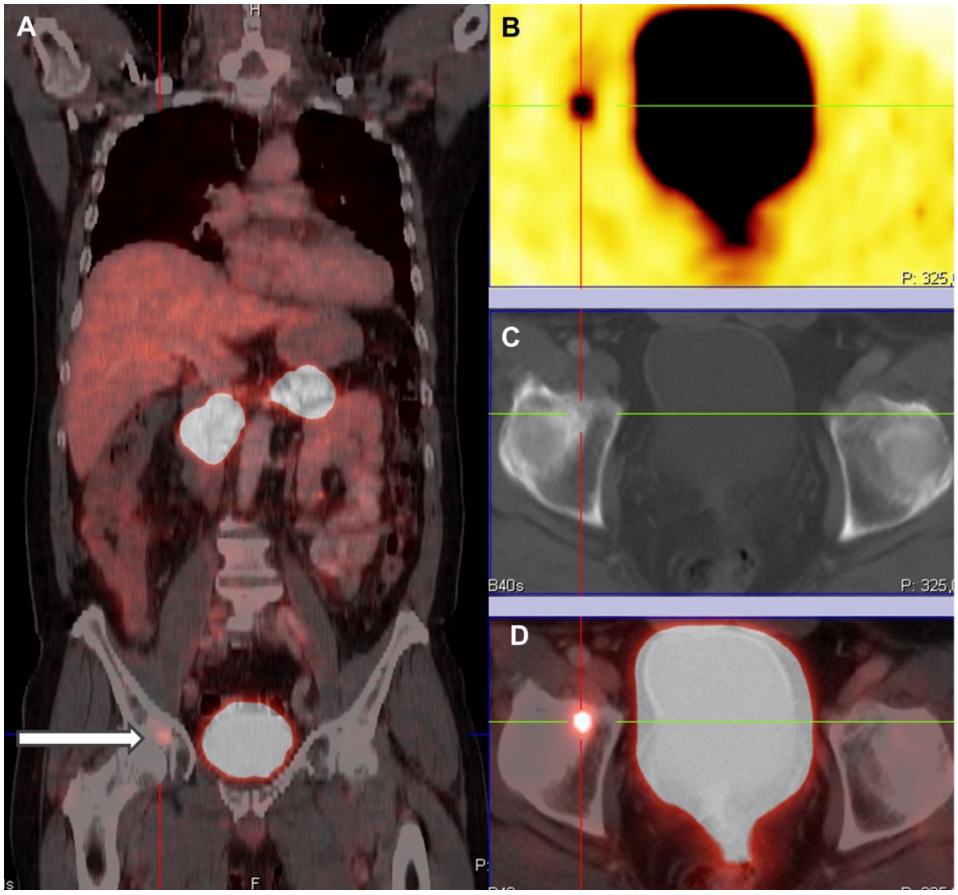
Data Reconstruction and Image Analysis

The CT transmission images were used for attenuation correction of the PET emission data. After scatter and attenuation correction, PET emission data was reconstructed using an attenuation-weighted ordered-subsets maximization expectation approach with 2 iterations and 8 subsets on 128×128 matrices and with a 5-mm Gaussian post-reconstruction filtering.

Image analysis of the CT was performed on a syngo viewing station by an experienced radiologist (more than 5 years of CT experience). The PET/CT images were assessed using E.soft (syngo based nuclear medicine software, Siemens Medical Solutions) by 2 experienced nuclear medicine physicians. Manually selected ROIs were automatically drawn on a single slice of the reconstructed PET/CT fusion images, for the measurement of SUV_{max} of different organs and structures, using the software provided by E.soft.



SUPPLEMENTAL FIGURE 4 Liver metastasis detected on [^{68}Ga]NeoBOMB1 PET/CT (**A**) MIP image, (**B**), transverse image through liver lesion in a patient with mildly differentiated acinar adenocarcinoma of the prostate, post endoscopic extraperitoneal radical prostatovesiculectomy with pelvic lymphadenectomy (PSA on the day of scan 12.6 ng/mL). Focal uptake of [^{68}Ga]NeoBOMB1 (SUVmax = 12.9) in segment 1 of liver, suggestive of a metastasis (blue arrows), which was not visualized on arterial phase of CT. However, a hypervascular lesion was seen on angiography of the liver with subsequent embolization and complete resolution of the lesion on follow-up. Physiological uptake is seen in the pancreas (green arrow), renal pelvises (white arrows), and bladder (orange arrow).



SUPPLEMENTAL FIGURE 5 Bone metastasis in the right acetabulum (arrow and crossbars) in a 64 year-old patient with prostate cancer (Gleason score 8) and a primary osseous metastasis. The patient on androgen deprivation therapy and was referred for [^{68}Ga]NeoBOMB1 PET/CT 2 years after the initial diagnosis with rising PSA (6.01 ng/mL at the time of presentation). [^{68}Ga]NeoBOMB1 PET/CT images: (**A**) PET/CT fusion coronal, (**B**) PET transverse, (**C**) corresponding CT transverse and (**D**) fusion PET/CT.

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