REVIEW ARTICLE

Recent Advances and Future Perspectives in Radiolabeled Antibody Fragments for Breast Cancer Molecular Imaging

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Abstract

Breast Cancer (BC) is the leading cause of cancer-related deaths in women and the most common cancer worldwide. It is classified based on its anatomical origin, the presence of Human Epidermal Growth Factor Receptor 2 (HER-2), and the presence of Estrogen Receptor (ER) and/or Progesterone Receptor (PR). Around 20% of breast cancers are HER-2 positive. While biopsy-based diagnoses are valuable in clinical settings, they have limitations in terms of sampling and interpretation. However, laboratory tests such as Immunohistochemistry (IHC) or Fluorescence In Situ Hybridization (FISH) are also limited, including being time-consuming, expensive, and requiring specialized equipment. Ongoing research and technological advancements aim to address the challenges associated with biopsy-based diagnoses and laboratory tests to develop more accurate and efficient methods for assessing HER-2 status. To this end, various radioactively labeled proteins and small compounds, such as single-chain variable Fragments (scFv), F(ab')2, affibody, and nanobody, have been developed to target HER-2 using molecular array techniques. These smaller targeted compounds offer improved image quality, shorter circulating half-life, and reduced immunogenicity compared to their larger counterparts. This is due to their better biodistribution, clearance, and stability. This study investigates the current understanding and ongoing efforts in utilizing antibody fragments for molecular imaging. The specific objectives were to evaluate the advantages of antibody fragments over full-length antibodies regarding biodistribution, clearance, and stability. Additionally, this study aims to assess the current knowledge and ongoing research in utilizing antibody fragments for molecular imaging.

Keywords: Breast Cancer; Antibody-Based Imaging; Radiolabel; Molecular Imaging; Nanobody.

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1. Introduction

Breast Cancer (BC) is the most common type among women and the second leading cause of worldwide cancer-related death. While BC rates are increasing globally, they are particularly high in industrialized nations for women. The survival rates for BC have greatly improved due to early detection and advancements in treatment. However, much must be done regarding prevention, early detection, and treatment [1, 2].

Breast cancer is classified according to its anatomical origin, which helps determine the disease's specific type and stage. The primary classification is based on where the cancerous cells first develop within the breast tissue. This information is crucial for clinicians to devise appropriate treatment plans and determine patients' prognoses [3, 4]. The most common type of breast cancer is ductal carcinoma, which originates in the milk ducts that carry milk from the lobules to the nipple. This type accounts for approximately 80% of all breast cancer cases. Another type is lobular carcinoma, which starts in the breast's milk-producing lobules. Although less common, lobular carcinoma requires a distinct approach due to its unique characteristics. Apart from ductal and lobular carcinomas, there are other less frequent types of breast cancer. These include inflammatory breast cancer, characterized by redness, swelling, and warmth in the breast; medullary carcinoma, known for its distinct borders and immune system response; and mucinous carcinoma, formed by mucus-producing cancer cells. Less common yet important classifications include tubular, papillary, and cribriform carcinoma. Each type has specific features and growth patterns, influencing the treatment options and outcomes. Furthermore, breast cancer can be categorized into different stages based on the size of the tumor, its spread to nearby lymph nodes, and whether it has metastasized to other parts of the body. The stages range from 0 to IV, with stage 0 indicating non-invasive cancer and stage IV representing metastatic cancer spreading to distant organs [5, 6]. Understanding breast cancer's anatomical origin and classification is crucial for healthcare professionals to provide personalized care and make informed treatment decisions. Identifying the specific type and stage of breast cancer allows physicians to customize therapies, including surgery, radiation, chemotherapy, targeted therapies, and hormone therapy, to maximize the chances of successful treatment and long-term survival [7-9].

More precisely, breast cancer is categorized based on its anatomical origin, expression of the Human epidermal growth factor receptor 2 (HER-2), and the presence of Estrogen Receptor (ER) and/or Progesterone Receptor (PR) [10, 11].

Generally, the diagnosis of breast cancer relies on the expression of HER-2, ER, and PR. Around 20% of BC patients have HER-2-positive status, linked to a poor prognosis [12, 13]. Trastuzumab, Epratuzumab, Trastuzumab-emtansine (a combination of antibody and drug), and Lapatinib (a tyrosine kinase inhibitor) are all approved treatments for HER-2-positive breast cancer. Histopathological investigations and ER, PR, and HER-2 status assessments are the only reliable biomarkers for predicting therapy response [14, 15]. However, the variability of BC tissues and the lack of reproducibility in studies limit the usefulness of immunohistochemistry research in determining HER-2 status. Newer approaches are being explored to overcome the limitations of immunohistochemistry in determining HER-2 status in breast cancer. One such approach is utilizing molecular methods like FISH and Polymerase Chain Reaction (PCR). These techniques provide a more accurate assessment of HER-2 gene amplification and expression levels. Targeted therapies are also being developed to address the heterogeneity of breast cancer tissues [16, 17]. For instance, new HER-2-targeted therapies such as Pertuzumab and Neratinib have shown promise in clinical trials. These drugs, in combination with existing treatments like Trastuzumab, offer improved outcomes for patients with HER-2-positive breast cancer [18, 19].

New diagnostic imaging methods have been developed to improve the early detection of breast cancer lesions using molecular imaging studies. These approaches aim to enhance our understanding of breast cancer pathology and enable the visualization and analysis of the disease in vivo [20, 21]. By harnessing the power of molecular imaging, researchers hope to significantly reduce the death rate from breast cancer by detecting it in its early stages and facilitating timely intervention, thereby improving patient outcomes [22, 23].

By combining pathology and molecular imaging, medical professionals can gain valuable insights into HER-2 expression throughout the body while prioritizing patient comfort and convenience. Numerous efforts have been made to discover and produce radiolabeled compounds that can enhance the diagnosis and treatment of breast cancer [24, 25]. Radiolabeled anti-HER2

antibody fragments offer a promising approach to improving the diagnosis and management of breast cancer. These efforts have resulted in the development of radiolabeled compounds targeting HER-2, an overexpressed protein in certain breast cancer cases. Attaching a radioactive label to these antibody fragments enables the visualization and quantification of HER-2 expression through non-invasive imaging techniques [26, 27]. This approach holds great potential for breast cancer diagnosis. By accurately assessing HER-2 expression throughout the body, clinicians can make informed decisions about treatment options, such as targeted therapies or personalized medicine. Moreover, this technique can assist in monitoring treatment response and identifying potential metastases or recurrence [28, 29].

Ten percent of BC cases are attributable to inherited mutations in specific genes, most notably the Breast cancer gene (BRCA)-1 and -2. BC risk factors are obesity and a high Body Mass Index (BMI), early sexual maturity, a first pregnancy before the age of 30, a history of ductal carcinoma, a family history of BC or Ovarian Cancer (OC), late menopause, and postmenopausal hormone treatment (mainly in white women) [11-15]. Several breast cancer subtypes are associated with different histology and prognoses (Table 1). The hormone receptivity of a breast cancer sample depends on the presence or absence of ER and PR expression, as well as the type of BC (lobular versus ductal). HER-2 is a protein that maps to chromosome 17q21; it belongs to the EGFR family of receptors for epidermal growth factors. Lymph node metastases, high-grade malignancies, and an increased risk of death are linked to HER-2 genetic alterations, making them a negative prognostic indicator [30, 31].

Most patients with invasive breast cancer are diagnosed with Infiltrating Ductal Carcinomas (IDCs), which account for about 80% of non-invasive carcinomas. In contrast, Invasive Lobular Carcinoma (ILC) is the most common form of lung cancer. Approximately 10% of breast carcinomas are also lobular carcinomas [32]. Around 70% of women with IDC show HR+/HER-2 incidences. It is essential to mention that ILCs are more common in postmenopausal women and tend to develop bilaterally. ER and PR are typically used in most cases. Ductal Carcinoma In Situ (DCIS) is a type of BC limited to the ducts where it originated. In rare instances, the DCIS phenotype may be observed with IDC on mammography or histopathology [19]. Morphological and cytological criteria are utilized for the classification of DCIS into subgroups. However, Lobular Carcinoma In Situ (LCIS) has a higher likelihood of being bilateral compared to Ductal Carcinoma In Situ (DCIS) (LCIS). Both DCIS and LCIS commonly exhibit HR+ and HER-2- negative characteristics. Young women with BRCA-1 mutation face an elevated risk of developing ductal breast cancer. Although inflammatory breast cancer is less prevalent than other forms, it poses a greater danger and has a worse prognosis [33]. Tubular, papillary, and mucinous breast cancer are rarer than Phylloides tumors [34].

Previously, it was demonstrated that HR+/HER-2- BC is the most common subtype [21]. The absence of hormone sensitivity and HER-2 expression characterizes the Triple-Negative Breast Cancer (TNBC) subtype. Approximately 12% of breast cancer cases occur in women with TNBC genes [22]. Non-Hispanic black women under 40 have the highest incidence and earliest detection rates of HER2-positive breast cancer. TNBC in women is more aggressive and typically diagnosed at a later stage compared to HR+/HER-2- cases. TNBC can be further classified into six subtypes: Basal-Like 1 (BL-1), Basal-Like 2 (BL-2), Mesenchymal Stem Cell-Like (MSL), mesenchymal (M), Luminous Androgen Receptor (LAR), and Immunomodulatory (IM) [35].

Histopathological markers, such as tumor size, metastasis, and axillary lymph node status exhibit breast cancer's biological characteristics and prognosis. Both the Tumor Microenvironment (TME) and disease progression play a role in tumor growth and lymph node involvement

Table 1. Main molecular subtypes of breast cancer

Type	Expression	Characteristics	Prognosis
Luminal A	HR+/HER-2-	Less aggressive than other subtypes	Good
Luminal B	HR+/HER-2+	Positive for Ki-67 or HER2; tends to be higher-grade	Fair
Triple-negative	HR-/HER-2-	The incidence of this disease is higher in black women than in white women; premenopausal women and those with BRCA1 mutations are at a greater risk	Poor
HER2-enriched	HR-/HER-2+	More aggressive than other subtypes	Poor

[36]. Among the early prognostic indicators of breast disease, the presence of microcalcifications is of utmost importance [25]. Microcalcifications in the breast are defined as calcium minerals smaller than 1 mm. The Breast Imaging Reporting and Data System (BI-RADS) of the American College of Radiology (ACR) describes breast microcalcifications radiologically [37]. Detecting micro- and macrocalcifications through mammography is a reliable predictor of developing ductal breast carcinomas [27]. Histopathological studies and the evaluation of ER, PR, Ki67, and HER-2 status are the only proven biomarkers for predicting therapy response [38]. Immunohistochemistry (IHC) is employed to assess the ER, PR, Ki67, and HER-2 status, aiding in prognosis determination and selecting the most suitable therapy strategy for BC patients [28].

This article explores research on radiolabeled antibody fragments as a tracer for breast cancer. It aims to provide a comprehensive overview of this molecular imaging technique's current understanding and potential. This study also explores the potential applications of antibody fragments in targeted therapy and diagnosis, aiming to utilize their improved biodistribution characteristics for more effective treatment strategies in various diseases.

2. Molecular Imaging

Molecular imaging, a medical technique, provides detailed images of cellular and molecular processes in the human body. Its ability to detect real-time changes in tissue structure is valuable for physicians in personalizing patient care. Furthermore, molecular imaging has the potential for non-invasive diagnosis and monitoring [39, 40]. It also aids drug delivery and enables experimentation with novel disease tracking and treatment methods. Molecular imaging advances our understanding of disease progression and treatment response. By visualizing the intricate workings of cells and molecules, this medical technique provides valuable insights into the underlying mechanisms of various diseases [41, 42]. With its ability to capture real-time changes in tissue structure, molecular imaging empowers physicians to make informed decisions about personalized patient care. One of the key advantages of molecular imaging is its potential for non-invasive diagnosis and monitoring [43, 44]. Medical professionals can accurately detect and track disease progression using specialized imaging agents without requiring invasive procedures. This reduces patient discomfort and allows for early detection and intervention, leading to improved treatment outcomes. Moreover, molecular imaging plays a vital role in drug delivery research [45, 46]. Scientists can optimize drug formulations and delivery methods by visualizing how drugs interact with target cells and tissues. This knowledge enables the development of more effective and targeted therapies, minimizing side effects, and maximizing therapeutic benefits. Additionally, molecular imaging opens up new possibilities for experimentation with novel disease tracking and treatment methods. Researchers can use this technique to investigate the efficacy of emerging therapies and develop innovative approaches to combat various diseases. By continuously pushing the boundaries of medical knowledge, molecular imaging paves the way for groundbreaking advancements in healthcare [47, 48].

Recent advancements in breast cancer molecular imaging have revolutionized diagnosing and treating this devastating disease. One significant breakthrough is the development of novel imaging techniques that enable the visualization of specific molecular targets within breast tumors. This breakthrough has provided researchers and clinicians with detailed information about the biological characteristics of the cancer, leading to more personalized and effective treatment strategies. One such advancement is Positron Emission Tomography (PET) imaging with radiotracers targeting molecular markers expressed by breast cancer cells. By injecting a radiotracer into the patient's bloodstream, PET scans can detect and map the distribution of these markers [36, 49]. This provides valuable insights into tumor metabolism, receptor status, and treatment response. This non-invasive approach has dramatically improved our ability to detect breast cancer at an early stage and monitor its progression over time. Another promising technique in molecular imaging is Magnetic Resonance Imaging (MRI) with contrast agents specifically designed to target breast cancer biomarkers. These contrast agents can bind to molecules or receptors expressed by cancer cells, enhancing the visibility of tumors on MRI scans. By combining the high spatial resolution of MRI with molecular targeting, clinicians can accurately assess tumor size, invasiveness, and the presence of

metastases [50]. This information guides treatment decisions and improves patient outcomes. Moreover, molecular imaging has opened doors for developing theranostic agents, which possess diagnostic and therapeutic properties (Figure 1). These agents can deliver targeted therapies directly to cancer cells while simultaneously providing real-time imaging of the treatment response. This approach holds great promise for personalized medicine, as it allows clinicians to tailor treatments based on the unique molecular characteristics of each patient's tumor [51-53].

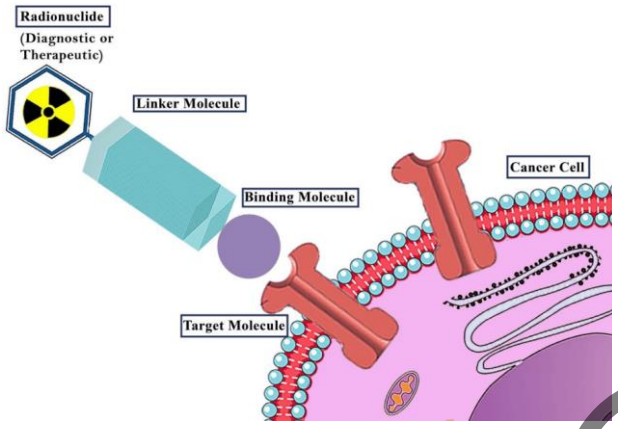

Figure 1. A schematic structure of targeted theranostic radiopharmaceuticals: In this targeted radionuclide, γ- or β+ emitters are used for diagnosis, and β- and α emitters are operated for diagnosis and treatment, respectively [54]

Approximately 20% of breast cancers are HER-2 positive (HER-2+). HER-2 is a receptor belonging to the ERbB/HER family of receptors, functioning as a transmembrane tyrosine kinase [55, 56]. HER-2 homo- or heterodimerization significantly impacts intracellular signaling, influencing various aspects such as apoptosis, proliferation, adhesion, and motility. To identify patients who may benefit from anti-HER-2 therapy, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) recommend testing all individuals with invasive breast cancer for HER-2 expression [35, 36]. IHC or FISH are the methods employed to determine HER-2 expression. In the case of breast cancer, HER-2 positivity can be indicated by either protein overexpression (3+ IHC status) or an elevated copy number of the HER-2 gene (identified as six or more by FISH). If conflicting results are obtained using the first method, the second method should determine the HER-2 status [57].

While biopsy-based diagnoses have proven valuable in clinical settings, they have limitations. One significant drawback is the potential for sampling error, as the small tissue sample obtained during a biopsy may not accurately represent the entire tumor. Moreover, inconsistency in interpretation and subsequent treatment decisions may arise due to inter-observer variability among pathologists [58].

Although biopsies have been clinically proven useful for diagnoses, the quality and representativeness of the biopsied material are crucial factors. However, despite their high frequency, it is notoriously challenging to determine the presence or absence of bone metastases in individuals with breast cancer using IHC [39, 59]. Significant challenges arise from biopsy-based methods, such as heterogeneity within and between tumors, as well as discrepancies in status between primary and metastatic tissues [60, 61]. The HER-2 status can also change the course of the disease or result from treatment-induced clonal selection [62]. To overcome these challenges, researchers propose collecting multiple samples from the same lesion, sampling at regular intervals to monitor disease progression, and obtaining samples from various metastatic sites [63, 64]. However, the significant hurdle in implementing these criteria lies in the associated morbidity of the biopsy procedure. Moreover, these tests can be time-consuming and costly, as they require specialized laboratory equipment and expertise. However, despite these limitations, IHC or FISH remains essential in determining HER-2 status and guiding treatment decisions for breast cancer patients.

Management of metastatic breast cancer still lacks a reliable, accurate, and non-invasive method for assessing HER-2 [65, 66]. Therefore, several ongoing research projects aim to develop in-vivo diagnostic procedures. PET using ^{18}F-Fluorodeoxyglucose (FDG) has revolutionized the imaging evaluation of breast lesions in managing breast cancer patients. Currently, PET and Computed Tomography (CT) scans are frequently performed together [67-69]. Consequently, ^{18}FDG-PET/CT analysis has become the established practice for evaluating the effectiveness of cancer treatment, as well as diagnosing, staging, and predicting patient outcomes in BC. The ^{18}F-FDG PET/CT scan is a sensitive technique for staging BC. This scan compassionately provides valuable insights into the scope and progression of BC [70].

Metastases were detected in the peritoneal lymph nodes, left liver lobe, and uterine cervix using FDG-PET/CT (Figure 2). The liver metastases exhibited notable ABY-025 uptake, while the peritoneal metastases showed moderate uptake, and the cervical metastases showed none. Only the liver revealed a positive result on IHC, whereas the other two locations yielded negative findings [70].

As an added benefit, this technique has the potential to provide valuable data for monitoring therapy response [71], which can be challenging when investigating multiple biopsies [72-74]. However, the primary method in this field for PET imaging is ^{89}Zr-trastuzumab. Research has demonstrated that ^{89}Zr-trastuzumab PET/CT analysis can detect HER-2 expression in the entire tumor burden of breast cancer patients, eliminating the need for repeated tissue samples to assess intra-patient heterogeneity of HER-2 status. In cases where the HER-2 status cannot be determined through standard diagnostic procedures [49], ^{89}Zr-trastuzumab PET scanning can assist clinicians in making decisions [75]. Additionally, Tamura described PET imaging using ^{64}Cu-DOTA trastuzumab in six HER-2+ breast cancer patients. The radiation dose from ^{64}Cu-DOTA trastuzumab was comparable to that from ^{18}F-FDG PET/CT [76]. With its favorable safety and efficacy profile, this radiopharmaceutical could diagnose primary and secondary breast cancer lesions and predict the biological response to anti-HER-2 monoclonal antibodies. This information could be valuable in choosing between anti-HER-2 antibodies and HER-2 tyrosine kinase inhibitors. When used as labels, ^{99m}Tc also ensures safety in regular settings [77]. Because of developments in biotechnology and the discovery of multifunctional nanoparticles that can be loaded with a wide range of therapeutic chemicals, novel therapeutic and diagnostic concepts are now at our disposal [78]. Radiolabeled trastuzumab and epratuzumab are promising tracers because they accumulate in HER-2+ tumor tissue. There is still time to make significant treatment adjustments; therefore, they could be more helpful. Since then, many other radiolabeled proteins and tiny compounds that specifically target HER have been developed [79], such as the Single-chain variable fragment (scFv),

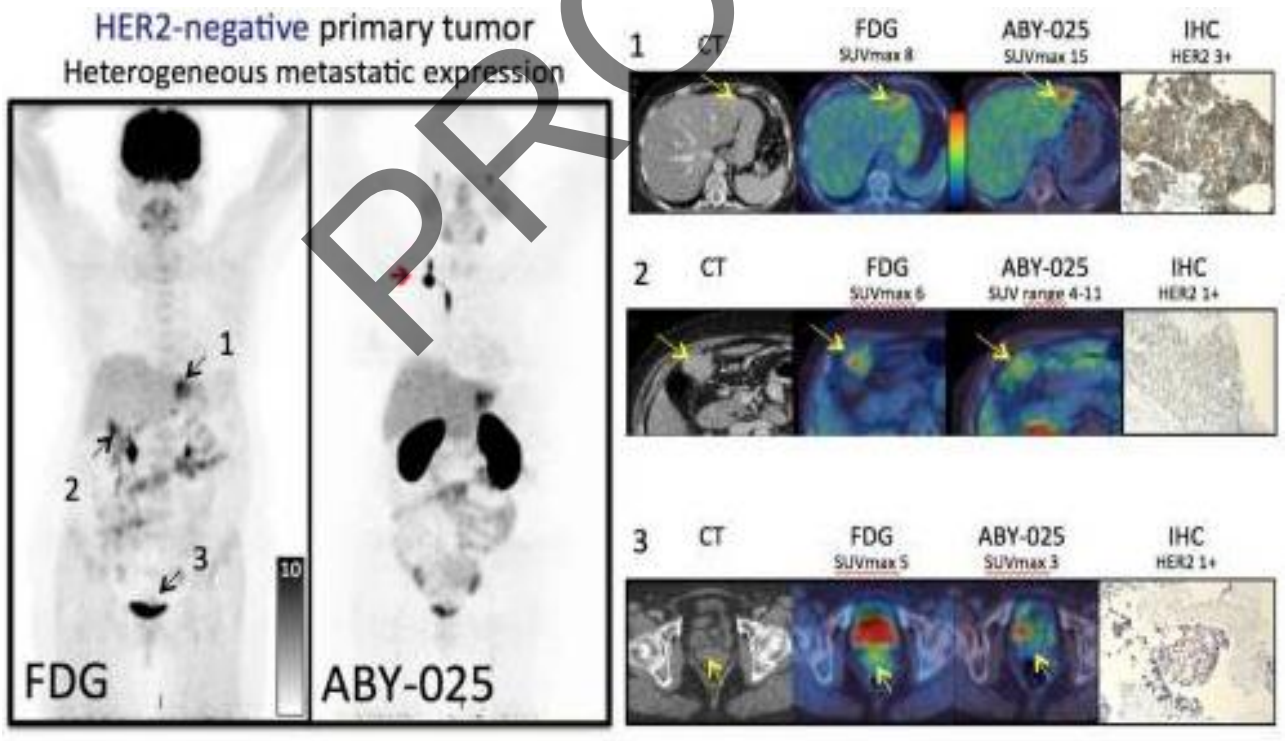

Figure 2. Demonstration of uptake levels in FDG and [^{68}Ga]ABY-025 in coronal PET imaging for breast cancer metastases: left lobe of the liver (1), peritoneum (2), and cervix (3) (left-sided images); The images consist of axial CT scans, PET with FDG, and PET/CT with [^{68}Ga]ABY-025, along with IHC to display HER2 expression in the liver (upper right images-1), peritoneal metastasis (middle right-2), and cervical metastatic cancer (lower right axial images-3). The results reveal high ABY-025 absorption in liver metastases, low uptake in peritoneal metastases, and no uptake in the cervical area. IHC confirms the ^{68}Ga findings [70]

F(ab')$_2$, affibodies, nanobodies, minibodies, and diabodies. Better image quality, a shorter circulation half-life, and less immunogenicity are all possible because of the improved biodistributions and clearance mechanisms displayed by small targeted agents. They undergo less complex chemical transformations, making them better suited and supporting decision-makers for routine therapeutic use [80, 81]. Bansch *et al.*, regarding the possibility of human epidermal growth factor receptor 2 (HER2) status in breast cancer using ^{89}Zr-trastuzumab PET, designed a test to see if this radiopharmaceutical compares to ^{18}F-FDG PET in cases when HER2 status cannot be defined by ordinary work up. Substantial results were obtained to determine the standard, as shown in Figure 3. They concluded that the effect of CTC HER2 status should be further investigated [75].

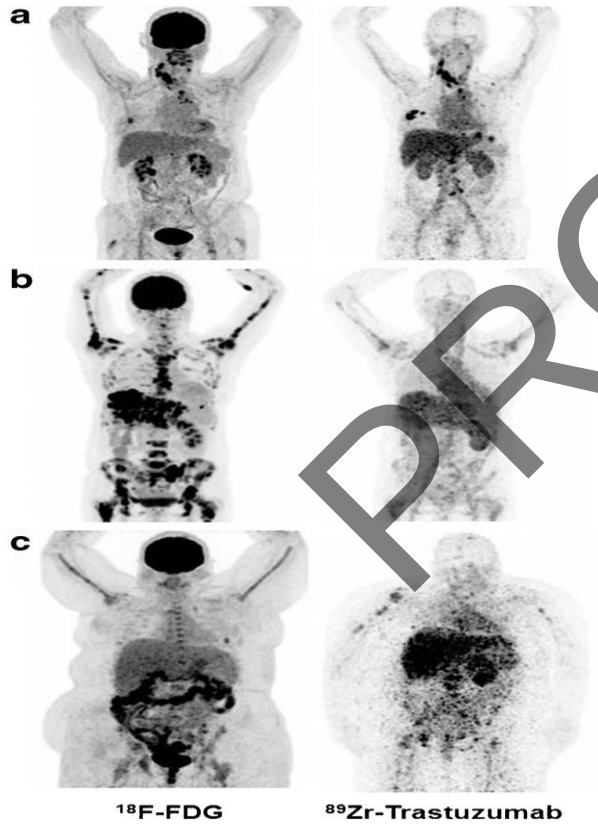

Figure 3. Comparison of PET images using two radiopharmaceuticals ^{18}F-FDG (left) and ^{89}Zr-trastuzumab PET scan (right) from three different patients with breast cancer to show the foci of metastasis: A right side of a patient with ^{89}Zr-trastuzumab PET scan shows that it was considered HER2-positive (a). In contrast, the middle image on the right illustrates an ^{89}Zr-trastuzumab HER2 PET scan that was considered HER2-negative (b), and the bottom right image of a PET scan displays ^{89}Zr-trastuzumab in an intermediate and ambiguous state (c) [75]

3. Fragments of Antibodies for Molecular Imaging of Breast Cancer

3.1. Fab and F(ab')2 Tracers

An antibody's Y-shaped structure consists of two antigen-binding fragments (Fab) and a crystallizable Fc fragment. The hinge region between the heavy chain's variable (Fc) and fixed (Fab) domains allows the antibody to form complexes with distantly located epitopes, such as dimers and trimmers [82, 83]. The Fab fragment of antibodies, which is responsible for binding antigens, is composed of the Light (L) chain's Variable (V) domain and the Heavy (H) chain's Constant (C) domain. The paratope, or antigen-binding site, is located in the V domain at the amino-terminal end of each monomer and comprises a series of Complementarity-Determining Regions (CDR). As a result, the Y-shaped antibody can recognize a specific antigen region to which it can bind. Both Fc and Fab fragments can be produced in a laboratory setting [84]. Papain can cleave an immunoglobulin monomer into two Fab fragments and one Fc fragment, while pepsin cleaves the protein into an F(ab')2 and a pFc' below the hinge. Several enzymes, including IdeS (Immunoglobulin degrading enzyme) from Streptococcus pyogenes, have recently been introduced for producing F(ab')2. IdeS cleaves the IgG sequence specifically at neutral pH [85].

Several radionuclides were tested on F(ab′)$_2$ and Fab fragments of trastuzumab and pertuzumab for imaging in animal models [86-91]. The labeling chemical affected the tracer uptake in both tumor and healthy tissue. However, all tested probes showed acceptable imaging within 24 hours of injection. In their study, Smith-Jones *et al.* compared ^{111}In-DOTA-(Fab)2-trastuzumab to ^{111}In-DOTA-trastuzumab [87]. Compared to ^{111}In-DOTA-trastuzumab, which had a maximum value of around 6.5 at 72 h, ^{111}In-DOTA-(Fab)2 achieved a tumor-to-blood standard uptake ratio (SUR) of 10 as early as 24 hours after injection. Therefore, F(ab′)2 and Fab fragments can mitigate the significant delay between the injection of fully radiolabeled antibodies and imaging (Figure 4). There have been reports of tumor-to-blood ratios as high as 19:8 after administering ^{64}Cu-NOTA-(Fab)2 epratuzumab [86].

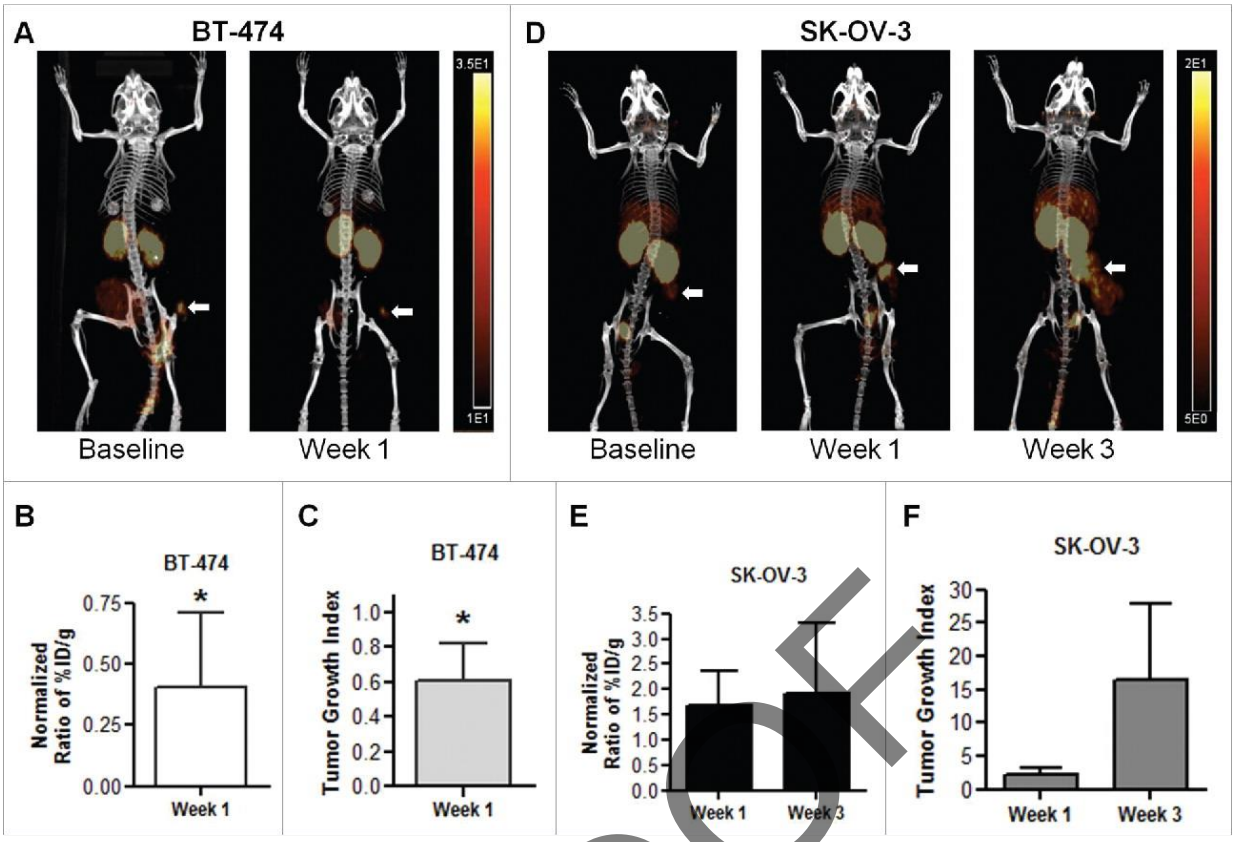

Figure 4. Micro PET/CT images of breast cancer and human ovarian cancer-induced tumor mice and their quantitative analysis were obtained at different time points following a specific therapy protocol using 64Cu-NOTA-pertuzumab F(ab')2 containing pertuzumab. In panel (A), the images were obtained 24 hours (left) and one week (right) after the injection of theranostic agents in human breast cancer following the initiation of trastuzumab treatment. Panel (B) presents the quantitative analysis of normalized uptake changes at the corresponding tumor site in BT-474 tumors after injecting 64Cu-NOTA-pertuzumab F(ab')2. Panel (C) displays the quantitative analysis of the tumor growth index after one week. Panel (D) shows the MicroPET/CT images captured 24 hours after administration of 64Cu-NOTA-pertuzumab F(ab')2 in mice bearing ovarian cancer xenografts at baseline, one week, and three weeks after the initiation of trastuzumab treatment. Panel (E) illustrates the corresponding changes in ovarian tumor uptake normalized relative to baseline using 64Cu-NOTA-pertuzumab F(ab')2. Finally, panel (F) presents the tumor growth index [86]

Beylergil *et al.* developed the PET imaging tracer ^{68}Ga-DOTA-F(ab′)2-trastuzumab to assess HER-2 expression in living organisms [61]. In this study, 15 women with BC (7 HER2- and 8 HER2+) participated. Seven of the eight patients with HER-2+ status had prior trastuzumab therapy, and one did not. Four of the eight patients showed tumor-targeting and good tolerance to ^{68}Ga-DOTA-F(ab′)2-trastuzumab. Additionally, ^{68}Ga-DOTA-F(ab′)2-trastuzumab effectively detected metastatic disease. The hypothesis suggests that high levels of circulating trastuzumab hindered tumor targeting by ^{68}Ga-DOTA-F(ab′)2-trastuzumab, explaining only 50% of the confirmed lesions (^{18}F-FDG established) [92].

3.2. scFv Tracers

When the variable sections of the heavy and light chains are combined, they form a single-chain variable fragment (scFv) that retains the original immunoglobulin's specificity. The development of specific scFv has enabled the creation of small imaging probes. In a study involving mice with SKOV-3 xenografts, it was found that 24 hours after injection with radioiodinated anti-HER-2 scFv 741F8 and C6.5, the tumor-to-blood and tumor-to-liver ratios were higher compared to imaging agents based on total antibodies [93, 94]. In another investigation, fluorescent Quantum Dots (QD) were utilized for passive and active targeting in a HER-2/neu+ BC model. Active targeting of tumors was achieved using

anti-HER-2/neu 4D5scFv antibodies, while contrast agents (QD-4D5scFv) consisted of 705 nontargeted QDs coated with Polyethylene Glycol (PEG). The concentration of the probes (QD-PEG and QD-4D5scFv) at the tumor site was examined using whole-body fluorescence imaging in vivo. Both passive and active administration techniques proved effective for tumor imaging, with QD-4D5scFv exhibiting a significantly stronger fluorescent signal than QD-PEG [95].

3.3. Affibody Tracers

Synthetic antibody fragments called "affibodies" have multiple applications in medicine and diagnosis. Affibodies that target staphylococcal Surface Protein A (SPA) adopt a stable three-helix structure consisting of 58 amino acids without disulfide bonds (SS-bond), which enhances their stability. Regarding molecular imaging, affibody molecules are well-suited due to their small size, fast turnover rate, high affinity, and ability to tolerate extreme temperatures and low pH [96, 97].

Clinical trials were conducted to evaluate the use of HER-2 affibodies, which demonstrated their effectiveness in targeting and visualizing tumors. The initial clinical trial of radiolabeled HER-2-affibody ABY-002 (DOTA ZHER2:342 pep2) in patients with advanced BC yielded promising results [98-101]. SPECT and PET images of ABY-002, labeled with indium-111 and gallium-68, were obtained within 2 hours post-injection. In most cases, the radioactive affibody confirmed the presence of lesions identified by 18F-FDG-PET. However, due to significant background uptake, only structures near the kidney and liver could be accurately recognized [102].

The ongoing advancement of affibodies has resulted in enhanced blood clearance and an increased ratio of background to tumor. Another affibody, ABY-025 (ZHER2:2891) (NCT01216033), has completed clinical trials. Patients with HER-2+ metastatic breast cancer exhibited a positive response to the ^{111}In-labeled affibody in terms of safety, bioavailability, and tumor targeting. Notably, high-contrast SPECT images were obtained between 4 and 24 hours after injection, despite the kidneys exhibiting the highest absorption in normal tissue, followed by the liver and spleen [79].

Two additional clinical studies were conducted using a 68Ga-labeled version of a similar affibody (NCT02095210, NCT01858116). To assess the impact on tumor uptake, two doses of the tracer were administered: 100 g and 500 g. PET scans performed 2 to 4 hours after injecting 500 g of 68Ga-ABY-025 demonstrated improved specificity and enhanced detection of metastasis [70, 103]. Currently, a phase II/III clinical trial (NCT03655353) is underway to evaluate the correlation between HER-2 expression, as measured by 68Ga-ABY-025 PET, and conventional histology obtained from relevant tumor samples.

HER-2-targeting affibody, ABH2, labeled with ^{99m}Tc, demonstrated a specificity of 60% overall in an open-label phase I clinical study (NCT03546478) involving HER-2+ BC patients. No Severe Adverse Events (SAE) were reported. Despite the liver significantly absorbing the radiotracer [104], high-contrast SPECT images were obtained at 1.5 and 4.5 hours after injection. Radiolabeled affibodies are currently being utilized in several ongoing clinical trials for molecular imaging of BC. For example, a phase I clinical trial (NCT04267900) is currently underway to evaluate the affibody HPArk2, tagged with ^{99m}Tc. Additionally, another open-label, non-randomized clinical investigation (NCT03827317) is assessing the efficacy of 18FGE-226 in measuring HER-2 expression in patients with metastatic BC [105].

The benefits of affibodies for molecular imaging are not without challenges. One example is the relatively low target affinity of affibodies, which is a serious concern [106]. To address this, modifications to the molecular design of the affibody would be necessary to reduce off-target interactions or background radioactivity [107]. Moreover, there are costs associated with creating radioactively labeled affibodies, and the production process faces challenges in scaling up. Additionally, the labeling procedures may increase lipophilicity, leading to unintended reactions with normal tissue and binding to blood proteins. Repeated therapeutic administration to patients also increases the risk of immunogenicity due to the bacterial origin of the protein scaffolds [107, 108].

3.4. Nanobody Tracers

As mentioned, antibodies have a Y shape consisting of two heavy and light chains. Sharks and camelids produce Heavy Chain Antibodies (HcAb) in addition to the more frequent light chain antibodies [109, 110]. Due to their lack of a light chain, HcAbs rely on the direct interaction between their variable domain and the Fc domain (CH2 and CH3) to bind antigens. In camels, the variable heavy domain is known as VHH, whereas in sharks, it is known as Variable New Antigen Receptor (VNAR) [111, 112]. The Very-small-H chain (VHH), sometimes called a Nanobody or a single-domain Antibody (sdAb), is physically and functionally comparable to the Fab segment in conventional antibodies despite its much smaller size (15 kDa). Because of their diminutive size, nanobodies undergo rapid renal clearance, resulting in a short biological half-life [113].

Because of its short half-life, fluor-18 is frequently used in PET imaging (110 min). Many different nanobodies with 18F tags have been developed and evaluated for use as PET tracers. Xavier *et al.* 18F-tagged the HER-2 nanobody and showed that it effectively targeted tumors in vitro. It was found that HER-2+ xenografts had high tumor-to-tissue ratios with selective uptake. The kidneys quickly excreted the 18F-anti-HER2-nanobody. The probe was also effective at scanning HER-2+ tumors when co-administered with trastuzumab, suggesting that the tracer may be useful for patient selection and therapy monitoring [114].

Keyaerts *et al.* conducted clinical trials of a ^{68}Ga-HER-2 nanobody to assess its safety, biodistribution, and dosimetry. ^{68}Ga- HER-2 nanobody was injected into 20 women with primary or metastatic BC, and dosimetry measurements were derived from images taken at 10-, 60-, and 90-minute post-injection. In order to evaluate the tumor-targeting capacity, the study included both primary and metastatic BC. Even an hour after injection, only 10% of the dosage was found in the blood, and there were no Significant Adverse Effects (SAEs) related to the tracer. Intestinal, liver, and renal background uptake was constant. Whereas initial tumors tended to have diffuse tracer accumulation, metastatic lesions tended to have more distinct, well-differentiated tracer accumulation [115]. To better understand how well nanobody tracers work in identifying brain metastases in BC patients, a phase II open-label, non-randomized, and single-center study (EudraCT 2015-002328-24, NCT03331601) [116] is now recruiting participants. Currently, there is a phase I clinical trial (NCT04040686) investigating the safety, dosimetry, and efficacy of ^{99m}Tc labeled anti-HER-2 nanobodies in BC diagnostic imaging and a phase II clinical trial (NCT03924466) investigating the correlation between image-based HER-2 quantification following uptake of ^{68}Ga- NOTA-2Rs15d in local or metastatic BC patients [117-119].

4. Conclusion

In conclusion, radiolabeled antibody fragments have the potential to revolutionize the diagnosis and treatment of breast cancer through their use as tracers in imaging. This molecular imaging technique allows for the specific targeting of cancer cells, monitoring of treatment response, and delivery of targeted therapy, making it a valuable tool in the fight against breast cancer. Further research and development in this field will undoubtedly lead to improved patient outcomes and a deeper understanding of breast cancer biology. Compared to Y-shaped antibodies, the radiolabeled antibody fragments used in molecular imaging of breast cancer, such as Fab, F(ab')2, scFV, affibodies, and nanobodies, demonstrate superior biodistributions and clearance. This results in improved image quality, a shorter circulation half-life, and reduced immunogenicity. However, concerns remain regarding off-target interactions and background radioactivity associated with these compounds. Therefore, more clinical trials are necessary to enhance their therapeutic use and optimize factors such as dosage, duration, sensitivity, and specificity.

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