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REVIEW



Current status and future expectations of nanobodies in oncology trials

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ABSTRACT

Introduction: Monoclonal antibodies have revolutionized personalized medicine for cancer in recent decades. Despite their broad application in oncology, their large size and complexity may interfere with successful tumor targeting for certain applications of cancer diagnosis and therapy. Nanobodies have unique structural and pharmacological features compared to monoclonal antibodies and have successfully been used as complementary anti-cancer diagnostic and/or therapeutic tools.

Areas covered: Here, an overview is given of the nanobody-based diagnostics and therapeutics that have been or are currently being tested in oncological clinical trials. Furthermore, preclinical developments, which are likely to be translated into the clinic in the near future, are highlighted.

Expert opinion: Overall, the presented studies show the application potential of nanobodies in the field of oncology, making it likely that more nanobodies will be clinically approved in the upcoming future.

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Nanobodies; single domain antibodies; oncology; clinical translation; diagnostics; therapy

1. Nanobodies: next-generation antibody mimetics with unique structural and pharmacological features

The immune system is designed to conduct a rapid and protective response toward harmful pathogens or cells and can be exploited in the design of targeted therapy and immunotherapies [1]. In particular, blood circulating IgG glycoproteins, produced by B lymphocytes, are of great interest due to their functional role in the humoral response [2]. Natural humoral immune responses can be exploited by man-made monoclonal antibodies (mAbs) that can bind to an antigen with high affinity and specificity [3]. As such, mAbs have been exploited in the clinic for several medical applications. Examples of medical applications with systemically administered mAbs or their conjugates include immune checkpoint blockade, radioimmunotherapy, and antibody-drug conjugates [3–5]. Structurally, conventional mAbs are composed of two identical heavy polypeptide chains and two identical light polypeptide chains [6]. Although mAbs are used in these medical applications, they are still large and complex molecules (~150 kDa) which can hamper successful targeting. Additionally, the production of monoclonal antibodies is associated with a high cost [7]. As such, a lot of research is going into employing antibody fragments, such as single chain variable fragments (scFv) or antigen-binding fragments (Fabs), which still bind to their target with a high affinity and specificity. However, these small antigen-binding fragments suffer

from decreased solubility and stability and a lower production yield [2,7,8]. Nanobodies (Nbs), also referred to as variable heavy domains of heavy-chain-only antibodies (VHHs) or single-domain antibodies, are alternative antibody fragments derived from the camelid heavy chain-only antibodies, which have gained interest in the last decade (Table 1) [9].

A Nb (12–15 kDa) is approximately 10 times smaller than a mAb (~150 kDa) and is composed of four conserved framework regions (FR1–4), forming the structure of the protein, and three variable complementarity determining regions (CDR1, CDR2 and CDR3), which compose the binding paratope (Figure 1). Despite containing only three CDRs (instead of six in mAbs), Nbs can still bind their target with affinities up to the nanomolar-picomolar (nM-pM) range [10]. Interestingly, the CDR3 region (and to a lesser extent also CDR1) of Nb is generally larger in size compared to the CDR3 of the mAb V_H domain, which is likely to compensate for the absence of the light chain in heavy chain only antibodies [8,10]. In addition, the paratope of Nbs is usually more convex, which allows them to bind discontinuous and cryptic epitopes, such as receptor-binding pockets, more easily than mAbs, where the paratope of mAbs is often flatter or concave [10–12]. Furthermore, their smaller size results in deep tissue penetration *in vivo* and enables efficient tumor targeting. However, this small size also results in rapid renal clearance and a short *in vivo* half-life time (minutes to hours) [12]. Nbs are also more soluble compared to other antibody-fragments, which is

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Article highlights

- Nbs are an interesting targeting moiety for multiple preclinical and clinical applications in the field of medicine.
- Due to their characteristics, Nbs are ideal diagnostic radiotracer vehicles to non-invasively image cancer or stromal cells.
- Several Nbs, inhibiting oncological or immuno-oncological pathways, are currently being investigated clinically.
- Nbs are used as therapeutic targeting agents to redirect toxic groups or T-cells toward tumors.

due to the lack of the V_L chain and the presence of more hydrophilic residues [13]. Furthermore, they are highly stable in different conditions (high temperature and acidic pH) and are resistant against proteolytic degradation [14]. Nbs are reported to be very low to non-immunogenic. This is due to the high homology between the Nbs and the V_H fragment of some human antibodies [15]. In addition, Nbs can be easily engineered including being humanized if necessary or reformatted to multivalent formats (bivalent, bispecific, trivalent, Fc fusion) that can enhance their apparent affinity (due to avidity effects) or change their functionality (by fusing the Nbs against one antigen with another against another antigen) [10,16]. Furthermore, the *in vivo* half-life time of Nbs can be extended in multiple ways. This includes increasing their size, via fusion to other proteins (albumin or Fc domain) or PEGylation, which will hamper renal clearance but also reduce tissue penetrations [17]. To partially circumvent this issue, Nbs have been fused to anti-albumin Nbs or domains, which results in a smaller increase in size and a smaller reduction in tissue penetration, while still enhancing the *in vivo* half-life time of these molecules [17,18]. In addition, it is possible fine tune the *in vivo* half-life time of these constructs by using Nbs or domains with different affinities toward albumin. Finally, Nbs can be easily produced in microbial systems, such as bacteria and yeast, which results in yields up to milligrams or even grams per liter of culture and enables low-cost production [12].

All of the aforementioned characteristics make Nbs an interesting alternative to mAbs or other antibody-fragments for preclinical and clinical applications in several diseases. This trend is also seen in the rise of (early) clinical trials using Nb-based diagnostics or therapeutics over the last decade. While oncology is an important research field in these clinical trials, it is important to mention that Nbs are also clinically tested in other research fields, including cardiology, rheumatology, and

infectious diseases. In this review, we will provide an overview of the most important oncological clinical trials using Nb-based diagnostics or therapeutics, while also highlighting current preclinical efforts which could end up in future oncological clinical trials (Figure 2).

2. Clinical trials and preclinical developments with nanobody-based diagnostics

Clinical diagnosis of tumors is most often performed via invasive biopsies, liquid biomarkers, and/or noninvasive imaging using X-ray computerized tomography (CT), magnetic resonance imaging (MRI), or positron emission tomography (PET) scans to detect metabolically active lesions taking up radioactive sugar [19,20]. However, these techniques either lack spatial overview (in the case of biopsies and liquid biomarkers) or provide little molecular information about the tumors. There is a clinical need to molecularly phenotype cancer, preferentially in a noninvasive manner and in all the lesions of the body [20]. Therefore, multiple radionuclide imaging tracers targeting molecular features of cancer cells or tumor-associated cells are being developed to aid in the diagnosis, molecular phenotyping, or assessment of therapy outcome of oncological disorders [21]. Different targeting moieties, including mAbs and mAb-fragments, are being used for these purposes. However, Nbs have gained quite some interest as targeting molecules for molecular imaging [22].

Indeed, to date, a large fraction of clinical trials involving Nbs evaluate their assessment as diagnostic tracers for radionuclide imaging (SPECT or PET) (Table 2). Their *in vivo* characteristics result in several advantages as compared to mAb-based diagnostics, including fast tumor penetration and short half-lifetime, which result in a fast and high target-to-background contrast, enabling same-day molecular imaging of patients with low radiation burden [21,22]. Indeed, the use of Nb-based diagnostics allows the labeling of Nbs with short-lived isotopes, such as ^{68}Ga and ^{18}F for PET or $^{99\text{m}}\text{Tc}$ for SPECT, resulting in a low radiation exposure (<5 mSv per scan), microdosing of the diagnostic tracer, and subsequent scanning as early as one hour post-injection [10,21,23]. Nb-based radiotracers currently under clinical investigation focus on the imaging of specific tumor types using cancer-specific antigens (e.g. HER2 and CLDN18.2) or immune-oncology responses (e.g. CD8, MMR, PD-L1, or PD-L2) and will be discussed in more detail below (Table 2).

Table 1. Overview of characteristics of monoclonal antibodies and nanobodies. Comparison of the different characteristics between monoclonal antibodies and nanobodies.

	mAbs	Nbs
Size	~150 kDa	~12–15 kDa
Structure	Heterodimer	Monomer
Mode of binding	Bivalent	Monovalent
Specificity	High	High
Affinity	nM–pM	nM–pM
<i>In vivo</i> half-life	Weeks	Minutes to hours
Tissue penetration	Slow	Fast
Development cost	High	Moderate
Paratope	Concave or flat	Convex
Clearance	Metabolized via liver	Excretions via kidneys

mAbs: monoclonal antibodies; Nbs: nanobodies.

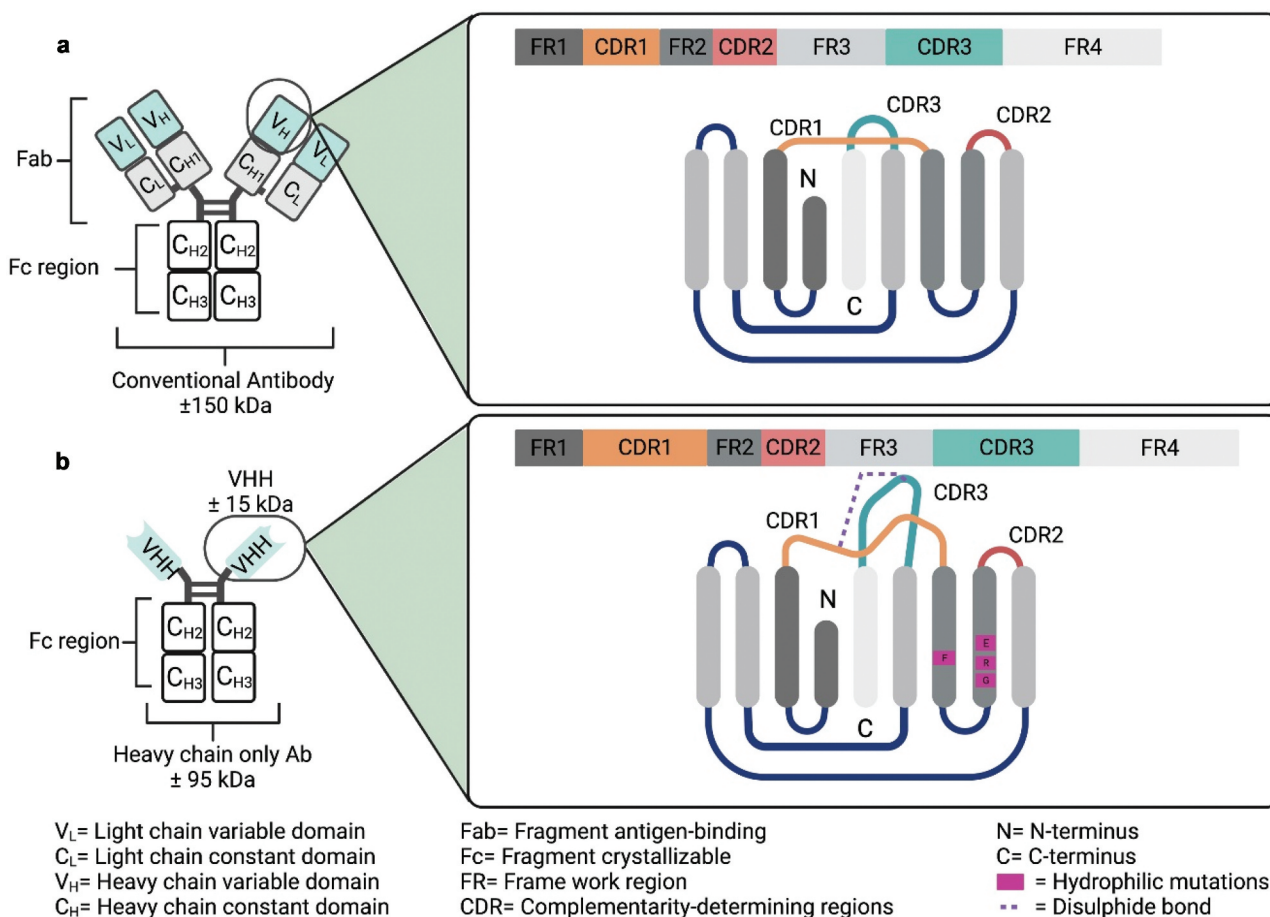


Figure 1. Schematic representation of the architecture of a conventional antibody, a heavy chain-only antibody and a nanobody (VHH). a) Structure of a conventional antibody consisting of two antigen-binding fragments (2× Fab), one fragment crystallizable (Fc region) and disulfide bonds in the hinge region. The architecture of the variable domain of the heavy chain of a conventional antibody (VH), which is composed of four framework regions (FR_{1,2,3,4} - gray) and three hypervariable regions (CDR_{1, 2, 3}) labeled with orange, red and blue, respectively. b) Structure of a heavy-chain-only antibody consisting of one Fc region and two variable domains (2× VHH) and disulfide bonds in the hinge region. The architecture of the VHH is shown, which is composed of four framework regions (FR_{1,2,3,4} - gray) and three hypervariable regions (CDR_{1, 2, 3}) labeled with orange, red and blue respectively, hydrophilic mutations in FR₂ and an extra disulfide bond between CDR₁ and CDR₃.

2.1. Nb-based clinical imaging tracers for cancer-specific antigens

The Human Epidermal Growth Factor Receptor 2 (HER2) is a transmembrane receptor tyrosine kinase that is overexpressed in several types of cancers, including breast, gastric, and ovarian cancers. Two decades ago, HER2 overexpression in these cancer types was correlated with a poor prognosis. Fortunately, HER2-targeting therapies, including mAbs, antibody-drug conjugates, and small molecules, have resulted in a favorable outcome for HER2-positive cancer patients. However, a correct diagnosis of the HER2 status of patients, using immunohistochemistry or fluorescence in situ hybridization, remains difficult due to inter- and intra-tumoral heterogeneity of HER2 expression, resulting in sampling bias [24,25]. Therefore, multiple groups have developed radiolabeled HER2-targeting moieties, including Nbs, for the noninvasive diagnosis of the HER2 status of patients. In 2016, our research group published a phase I study with a ⁶⁸Ga-labeled anti-HER2 Nb for PET/CT in patients with primary or metastatic HER2-positive breast carcinomas (Figure 3a,b) [26]. This was the first clinical study where a Nb-based diagnostic tracer was tested in

patients. Administration of the tracer did not result in adverse events and did not elicit anti-drug antibody levels in the blood of these patients. Both primary tumors and metastatic lesions could be visualized using PET/CT, with imaging being possible 60–90 min post-injection. Based on the successful phase I study, the ⁶⁸Ga-labeled anti-HER2-Nb is currently being investigated in two phase II studies, including a repeatability study (NCT03924466) and a study to determine the possibility to visualize brain metastasis (NCT03331601) in breast carcinoma patients. Similarly, another HER2-targeting Nb (NM-02 or RAD201) has been tested as a diagnostic tracer upon ^{99m}Tc-radiolabeling followed by SPECT/CT imaging in two separate clinical trials (NCT04040686 and NCT04674722) [27]. Similar results as seen with our study were observed, with no observable adverse events and a high uptake in HER2-positive lesions. Finally, a novel anti-HER2 Nb (^{99m}Tc-MIRC208) was recently developed and tested preclinically upon ^{99m}Tc-radiolabeling. ^{99m}Tc-MIRC208 was able to indirectly determine the accessibility of HER2 for Trastuzumab in tumor-bearing mice, which resulted in the initiation of clinical testing (NCT04591652). Preliminary imaging results of two breast

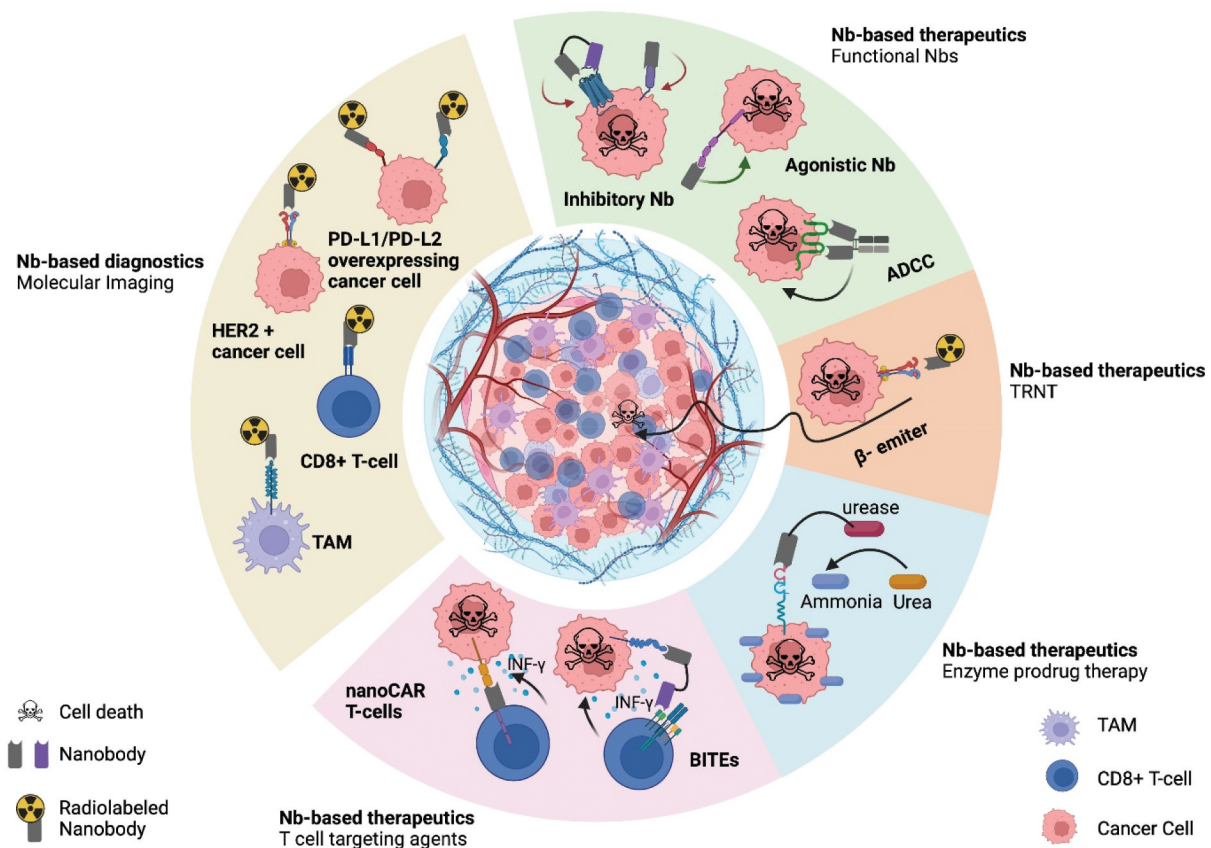


Figure 2. Overview of oncological clinical trials with Nb-based diagnostics and therapeutics. Schematic representation of the different applications cited in this review paper. Nb-based diagnostics are currently focusing on SPECT- or PET-imaging of specific tumor types expressing cancer-specific antigens (HER2+ tumor cells) or immune-oncology responses (CD8+ T-cells, PD-L1/PD-L2, or tumor-associated macrophages (TAM)). Nb-based therapeutics are represented based on their mode of action: functional Nbs (inhibitory Nb, agonistic Nb or antibody-dependent cell-mediated cytotoxicity (ADCC)), targeting agents to deliver toxic agents (targeted radionuclide therapy (TRNT) and enzyme prodrug therapy), bispecific T-cell engagers (BiTcs) and Nb-based Chimeric antigen receptor (CAR)-T-cells (nanoCAR-T cells).

cancer patients using ^{99m}Tc -MIRC208 are again in line with the two previously described tracers [28]. The same group reported the generation and first-in-human clinical trial of the anti-HER2 ^{18}F -labeled RESCA-MIRC213 Nb, which also shows similar results [29].

Another Nb-based diagnostic that is currently being clinically tested, is the ^{68}Ga -labeled anti-Claudin 18.2 Nb ACN376 (NCT05436093). Claudin 18.2 is a component of tight junctions and is overexpressed in several cancers, including pancreatic cancer and gastric cancer [30]. Currently, recruitment of patients is ongoing, and no results have been published yet.

2.2. Nb-based clinical imaging tracers for immune-oncology

Immunotherapy has revolutionized the oncology field and has become the standard of care for some cancer types. Some examples of immunotherapy include mAb-based immune checkpoint inhibitors targeting PD-1, PD-L1, and CTLA-4, but also bispecific antibodies, antibody-cytokine-fusions, Chimeric Antigen Receptor (CAR)-T-cells, and other adoptive cell therapies. However, variations in immunotherapy responsiveness and side effects between patients limit the broad applicability

and success of immunotherapies [31]. As such, noninvasive imaging of immune cell populations or immune activation/inhibition markers, to monitor anti-cancer immune responses, has gained a lot of interest [32]. This has resulted in the development of multiple (pre)clinical diagnostic tracers targeting immune checkpoints (PD-1, PD-L1, CTLA-4, TIGIT, and LAG-3) and different immune cell populations (CD4+ T-cells, CD8+ T-cells, and macrophages).

Programmed death-ligand 1 (PD-L1) is an immunoinhibitory molecule that is overexpressed in several tumor cells and inhibits T-cell activation. To date, multiple PD-L1-targeting mAbs (Atezolizumab, Durvalumab, and Avelumab) have been approved as treatment for several types of cancer, including urothelial carcinoma, melanoma, and Non-Small Cell Lung Carcinoma (NSCLC) [33]. Current eligibility criteria using PD-L1 immunostaining of tumor biopsies suffer from the same sampling biases as HER2 status prediction described above [34]. As an alternative to predict therapy response in patients, several PD-L1-targeting radiotracers have been developed [35]. A ^{99m}Tc -labeled anti-PD-L1 Nb (NM-01), which showed subnanomolar affinity to human PD-L1 and successful *in vivo* targeting in PD-L1-positive HCC827 xenograft mice, was tested on NSCLC patients in an early phase I clinical trial in 2019. No

Table 2. An overview of clinical trials using nanobody-based diagnostics. An overview of the different nanobody-based diagnostics which have been and/or are being tested in clinical trials.

Nanobody tracer	Company/institute	Disease	Target	Clinical trial	Phase	Status	Ref
⁶⁸ Ga-NOTA-Anti-HER2 VHH1	Vrije Universiteit Brussel/UZ Brussel	Breast carcinoma Metastatic breast carcinoma Locally advanced breast cancer Brain metastasis in breast cancer	HER2	012 - 001,135-31(EudraCT) NCT03924466	I II	Completed Recruiting	26 /
^{99m} Tc-NM-02/ RAD201	Nanomab/Radiopharm	Breast cancer	HER2	NCT04040686 NCT04674722	I I	Completed Recruiting	27 /
^{99m} Tc-MIRC208	Peking University Cancer Hospital and Institute	HER2-positive cancer patients	HER2	NCT04591652	NA	Recruiting	28
¹⁸ F-RESCA-MIRC213	Peking University Cancer Hospital and Institute	HER2-positive breast cancer patients		2021KT108 (Beijing cancer hospital ethical approval)	I	Completed	29
⁶⁸ Ga-ACN376	Peking University Cancer Hospital and Institute	Solid tumors	CLDN18.2	NCT05436093	I	Recruiting	/
^{99m} Tc-NM01	Nanomab	NSCLC NSCLC Melanoma Advanced NSCLC Solid tumor	PD-L1	NCT02978196 NCT04436406	I I	Completed Recruiting	36 /
⁶⁸ Ga-NOTA-SNA002	Smart Nuclide	Solid tumor	PD-L1	NCT04992715 NCT05490264	II I	Recruiting Recruiting	37 38
⁶⁸ Ga-THP-APN09	Peking University Cancer Hospital and Institute	Solid tumors	PD-L1	NCT05156515	I	recruiting	/
⁸⁹ Zr-Envafoimab	Alphamab	Advanced solid tumors	PD-L1	NCT03638804 NCT04977128	pilot	Recruiting	39
⁶⁸ Ga-MIRC415	Peking University Cancer Hospital and Institute	Solid tumors	PD-L2	NCT05803746	I	Recruiting	/
⁶⁸ Ga-NODAGA-SNA006	Smart Nuclide	Lung cancer	CD8	NCT05126927	I	Completed	42
⁶⁸ Ga-NOTA-Anti-MMR VHH2	Vrije Universiteit Brussel/UZ Brussel	Malignant solid tumor Breast cancer Head and neck cancer Melanoma	CD206	NCT04168528 NCT04758650	I/IIa	Recruiting	47

NA: Not applicable; NSCLC: Non-Small Cell Lung Cancer.

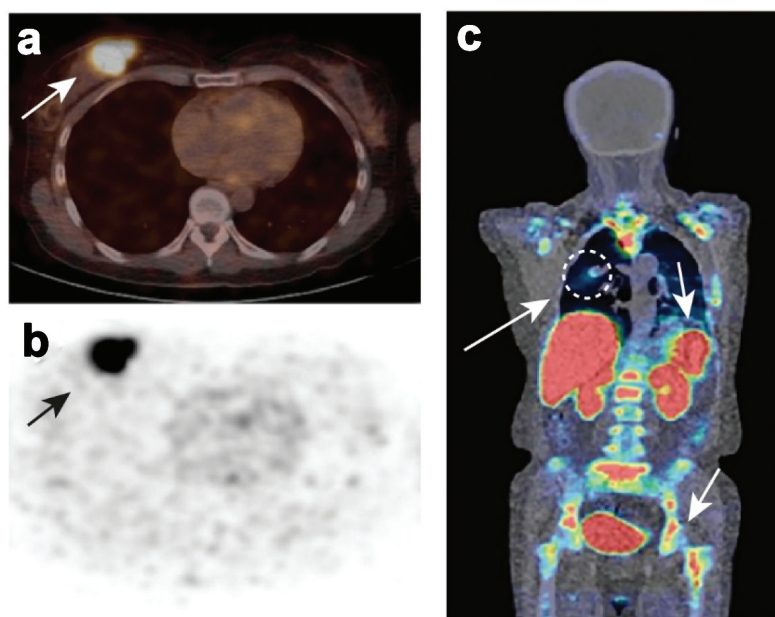


Figure 3. PET/CT imaging of cancer and immune cells using Nb-based diagnostics. A-B) example of noninvasive imaging of a HER2+ breast carcinoma tumor using ⁶⁸Ga-NOTA-Anti-HER2 VHH1. HER2-expression could be visualized with ⁶⁸Ga-NOTA-Anti-HER2 VHH1 in a primary breast carcinoma lesion (arrow) on PET/CT images (a) and PET images (b). This image was originally published in J Nucl Med: M. Keyaerts, C. Xavier, J. Heemskerk et al. Phase I study of ⁶⁸Ga-HER2-nanobody for PET/CT assessment of HER2 expression in breast carcinoma. J Nucl Med. 2016;57(1):27–33. © SNMMI. c) Example of noninvasive PET/CT imaging of CD8+ T-cells in a lung cancer patient 60 mins post injection of ⁶⁸Ga-NODAGA-SNA006. CD8+ T-cells could be visualized in the lung tumor (circle and arrow) and immune-rich organs, including bone marrow and spleen (arrows). This image was originally published as Wang, Y. et al. Pilot study of a novel nanobody ⁶⁸Ga-NODAGA-SNA006 for instant PET imaging of CD8+ T-cells. Eur J Nucl Med Mol imaging 49, 4394–4405 (2022).

drug-related adverse reactions were observed with an ideal imaging point two hours post injection [36]. Furthermore, PD-L1 expression in primary tumors and metastatic lesions could be visualized. The ^{99m}Tc-labeled NM-01 is currently

being tested in another phase I clinical trial (NCT04436406), where the relationship between tracer uptake and metabolic response to anti-PD-1/PD-L1 therapy is determined, and a phase II trial (NCT04992715) in

metastatic NSCLC patients [37]. In parallel, other PD-L1-targeting Nbs such as Nb109 (rebranded to SNA002) and APN09 are currently being tested as ^{68}Ga -labeled PET tracers in an early phase I study, focusing on the safety and uptake in patients with solid tumors (NCT05490264 and NCT05156515) [38]. Finally, a therapeutic anti-PD-L1 Nb-Fc fusion protein KN035/Envafolimab (see more information in section 3.1) is currently being tested as a diagnostic PET tracer in clinical trials. A ^{89}Zr -labeled version, which was previously validated in mice and non-human primates, is currently being tested in two pilot clinical trials (NCT03638804 and NCT04977128) to evaluate the safety and biodistribution in PD-L1-positive solid tumors. However, no further information is known yet about these studies [39]. Recently, a phase I study (NCT05803746) to evaluate the diagnostic potential of a ^{68}Ga -labeled Nb (MIRC415) targeting PD-L2, another immunoinhibitory molecule expressed in cancer cells, has been initiated with the aim of predicting/following up the response to PD-1/PD-L1 therapy.

Cytotoxic CD8⁺ T-cells are believed to be the main effector cells during an anti-cancer immune response [40]. As such, imaging of (intratumoral) CD8⁺ T-cell dynamics during immunotherapy could allow the prediction or follow-up of the therapy response [41]. To date, multiple imaging tracers, including mAbs, minibodies, and Nbs, have been tested, both preclinically and clinically, with promising results. Currently, an early phase I study (NCT05126927) with a ^{68}Ga -NOTA-CD8-targeting Nb (SNA006) is being performed. Initial preliminary results, whereby three lung cancer patients were imaged, showed no adverse events after tracer administration. Moreover, uptake of the tracer was seen in the lymphoid-rich organs, including the spleen and bone marrow, as well as the tumor, already 15 min post injection (Figure 3c). Furthermore, the uptake levels of the tracer in the tumor differed between patients, which could be linked to CD8⁺ T-cell density in these tumors via immunohistochemistry. However, additional patient data should further validate these promising results [42].

Diagnostic imaging of protumoral immune cells, such as subsets of tumor-associated macrophages (TAMs), has also gained interest [43]. One subclass of TAMs is the CD206 or macrophage mannose receptor (MMR)-positive TAMs. In multiple tumor types, the presence of CD206⁺ TAMs in the tumor microenvironment (TME) is linked with an overall poor survival, indicating the prognostic value of these TAMs [44]. Our group has developed a cross-reactive CD206-targeting Nb tracer (^{68}Ga -NOTA-Anti-MMR VHH2), which could visualize CD206⁺ macrophages upon ^{68}Ga -labeling in tumor-bearing mice [45,46]. This tracer is currently being tested in a phase I/IIa clinical trial to evaluate the safety, biodistribution, radiation dosimetry, and tumor targeting in breast cancer, head and neck cancer, and melanoma patients (NCT 04168528) [47] and phase II trial to evaluate the clinical potential in oncological disorders, cardiovascular atherosclerosis, and cardiac sarcoidosis (NCT04758650).

2.3. Preclinical developments of Nb-based imaging tracers

While currently only a few Nb-based diagnostic tracers are clinically tested, this will most likely grow in the coming years. In the case of radionuclide imaging, several Nb-based tracers targeting other cancer-specific antigens have been/are being developed preclinically and could be translated toward the clinic. Some examples include Nb-based diagnostics targeting the epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA), HER3, CD20, Mesothelin (MSLN), Prostate-Specific Membrane Antigen (PSMA), and Hepatocyte growth factor [10]. These tracers allowed the visualization of antigen-expressing tumors via SPECT or PET imaging. In addition, efforts are being made to allow noninvasive imaging of the TME or stroma using radiolabeled Nbs. Examples include imaging of myeloid cells (using Nbs targeting CD11b, MHC-II, or SIRPα), LAG-3 positive T-cells, or the extracellular matrix and neovasculature using a Nb targeting alternatively spliced E11B domain of fibronectin [10,48–50]. While some of these examples entail Nbs targeting the murine homolog, and as such do not allow clinical translation, they show the interest and promise of noninvasive nuclear imaging of cancer-specific membrane antigens and TMEs.

Besides nuclear imaging, efforts are being put into the development of fluorescently labeled Nb-based tracers. On a preclinical level, fluorescently labeled or tagged Nbs have already been widely used as research tools (biosensors) to detect and track specific proteins or protein conformations [51,52]. However, fluorescently labeled Nbs are also being used for translational purposes such as *in vivo* imaging applications including image-guided surgery [53]. Here, Nbs are labeled with fluorophores in the near-infrared region (e.g. IRDye800CW) and allow the visualization of specific tissues, for instance tumor or surrounding healthy tissue, to aid the surgeon in the complete resection of tumors during surgery [54]. To date, multiple preclinically evaluated fluorescently labeled Nbs have been described. These include Nbs targeting HER2, CAIX, EGFR, and CEA for intraoperative imaging of primary breast, ovarian, or colorectal tumors and their metastases [10,53]. While the use of fluorescently labeled Nbs significantly aided in a more complete resection of tumors, this technique can only be applied to superficial lesions, or lesions lying 1 cm deep in tissue due to limited tissue penetration of fluorescence [53]. To overcome this drawback, focus is being put on the design of bimodal Nb-based tracers whereby fluorescence is combined with radioactivity to first allow tumor localization via radionuclide imaging and/or gamma probe detection, followed by fluorescently image-guided surgical removal [53,55]. To date, all work involving fluorescently labeled Nb-based diagnostic tracers has been preclinical. However, clinical translation seems only to be a matter of time, since multiple groups are pursuing this.

To date, random (e.g. NHS-ester-based) conjugation with chelators or fluorophores, making use of the free lysines present in the Nbs, is often used to generate these Nb-based diagnostic tracers [56]. While this labeling technique is fast,

easy and relatively cheap, it also results in a heterogeneous mixture of labeled proteins and can result in loss of affinity if lysines are present in or close to the paratope of the Nbs [56,57]. As a result, a trend toward site-specific labeling of Nb-based tracers is noticeable. Some well-known examples of site-specific labeling include maleimide conjugation (making use of a C-terminal cysteine) and sortase A conjugation (making use of a c-terminal LPXTG sequence) [58]. Making use of these site-specific labeling strategies ensures a homogenous population of labeled Nbs, each containing one chelator or fluorophore in their C-terminus which does not interfere with antigen binding, is obtained. Some of the Nb-based tracers described above are already making use of these site-specific labeling strategies. However, multiple other site-specific labeling strategies are currently being preclinically investigated and could also aid in the clinical translation of Nb-based diagnostic tracers [57,58].

3. Clinical trials and preclinical developments with nanobody-based therapeutics

The reporting of studies describing therapeutic Nbs for oncological, but also for a variety of other diseases, has grown exponentially over the last decades [59–62]. In 2018, the first Nb-based therapy, Caplacizumab, for the treatment of acquired thrombotic thrombocytopenic purpura, was clinically approved [63]. Since then, other Nb-based therapies have been approved, such as Ciltacabtagene Autoleucel (US and conditionally in EU), a Nb-based CAR-T-cell therapy, Ozoralizumab (Japan), a Nb compound against rheumatoid arthritis, and Envafoimab (conditionally in China), a Nb-based immune checkpoint inhibitor [64–69]. In addition, several other therapeutic Nbs are currently being tested clinically, most of them in oncology (Table 3). These Nb-based treatments can be divided based on their mode of action (MoA), namely: (i) Nbs having a functional effect, (ii) Nbs used as targeting agents to deliver toxic agents, (iii) Nbs used as bispecific T-cell engagers (BiTEs) and (iv) Nb-based CAR-T-cells (nanoCAR-T cells) [59–61].

3.1. Functional therapeutic nanobodies in oncology trials

Multiple receptors, (over)expressed on cancer cells or tumor-associated cells, influence cellular signaling pathways in these cells and, as such, influence oncogenic processes such as proliferation, angiogenesis, pro-tumoral inflammation, and changes in the TME. Therefore, multiple researchers have been developing and testing therapeutics, including Nbs, that can influence the activity of these receptors as potential anti-cancer agents. A Nb can function as an agonist, antagonist, and/or inverse agonist depending on the targeted protein and epitope. Moreover, a Nb can exert its function in an orthosteric or allosteric manner [61].

To date, several classes of anti-cancer Nb-based therapeutics have been described at a preclinical level, including Nbs targeting tumor antigens (e.g. EGFR, HER2, VEGFR, c-Met, CEACAM6, CXCR4, ACKR3, and US28), Nbs acting as immune checkpoint inhibitors (e.g. PD-L1, CTLA-4, TIM-3, and CD47), or

Nbs neutralizing pro-tumoral cytokines (IL-23, TNF- α) [59]. However, to our knowledge, only a few Nbs, exerting a functional effect, are being/have been tested in an oncological clinical trial.

ALX-0651 is a biparatopic Nb, consisting of two monovalent Nbs targeting different epitopes of the chemokine receptor CXCR4, which was developed by Ablynx/Sanofi. CXCR4 facilitates hematopoietic stem cell trafficking, via binding of CXCL12 to CXCR4 [70]. Moreover, CXCR4 is overexpressed in multiple types of cancers and linked to tumor progression and metastasis [71]. ALX-0651 inhibits CXCR4 function and was envisioned to act as a potential therapeutic for non-Hodgkin's lymphoma or multiple myeloma (MM). It was tested in healthy volunteers in a phase I study (NCT01374503) to evaluate the safety and tolerability of the Nb. While the study proved the ability of ALX-0651 to target CXCR4, preliminary data indicated it is unlikely for ALX-0651 to outcompete standard care options and Ablynx discontinued this clinical trial [72].

Death receptor 5 (DR5) is a receptor that upon activation via binding of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), initiates caspase-induced apoptosis in tumor cells [73]. As such, several agonistic anti-DR5 therapeutics have been clinically tested. However, despite promising preclinical data, clinical studies with these agonistic therapeutics showed insufficient clustering and DR5 activation [74]. TAS266 is an agonistic tetravalent humanized Nb, which clusters DR5 receptors more easily than agonistic anti-DR5 mAbs, resulting in enhanced potency and stronger anti-tumor effects in different preclinical tumor models [75]. Based on these results, a phase I clinical trial (NCT01529307) in patients with advanced solid tumors was initiated. In total, four patients received at least one dose of TAS266 at 3 mg/kg dose level via intravenous (i.v.) injection. However, hepatotoxicity, with increased aspartate aminotransferase and/or alanine aminotransferase levels, was observed in three out of four patients [75]. This was striking as this was not observed in preclinical safety studies using cynomolgus monkeys and *ex vivo* human hepatocyte assays. Based on these results, the study was terminated early, and the development of this Nb stopped.

DR30303 is an anti-Claudin18.2 Nb, which has been humanized and fused to an engineered Fc fragment, resulting in enhanced antibody-dependent cellular cytotoxicity (ADCC). Preclinical studies in gastric and pancreatic xenograft mouse models showed enhanced anti-tumoral potency of DR30303 compared to anti-Claudin18.2 Zolbetuximab and synergistic effects with chemotherapeutic agents [76–78]. DR30303 is currently being evaluated in an open-label phase I study (NCT05639153) to evaluate the safety, pharmacokinetics, tolerability, and efficacy in patients with advanced solid tumors [79].

BI 836,880 is a half-life extended (via genetic fusion to an anti-albumin Nb) humanized bispecific Nb, targeting both vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang-2), key factors involved in tumor angiogenesis [80]. In 2016, two phase I studies (NCT02674152 and NCT02689505) were initiated to determine the maximum tolerated dose and ideal dose for phase II studies [81,82]. When looking

Table 3. An overview of clinical trials using nanobody-based therapeutics. An overview of the different nanobody-based therapeutics that have been and/or are being tested in clinical trials.

Nanobody	Company/ institute	Disease	Target	MoA	Clinical trial	Phase	Status	ref
ALX-0651	Ablynx/Sanofi	NHL, MM	CXCR4	Inhibition	NCT01374503	I	Discontinued	/
TAS266	Ablynx/Sanofi	Advanced solid tumor	DR5	Agonism	NCT01529307	I	Terminated	75
DR30303	Doer Biologics	Advanced solid tumor	Claudin 18.2	ADCC	NCT05639153	I	Recruiting	79
BI 836,880	BoehringerIngelheim Ingelheim	Advanced solid tumor	VEGF + Ang-2	Inhibition	NCT02674152 NCT02689505 NCT03972150	I	completed	81–83
Envafohimab/ KN035	Alphamab	Advanced solid tumor Advanced carcinoma Advanced solid tumor dMMR/MSI-H advanced solid tumor	PD-L1	Inhibition	NCT02827968 NCT03101488 NCT03248843 NCT03667170	I I I II	Completed Completed Completed Recruiting	86 / 84 68
KN044	Alphamab	Advanced solid tumor	CTLA-4	Inhibition	NCT04126590	I	Recruiting	/
KN046	Alphamab	Multiple disorders	PD-L1 + CTLA-4	Inhibition	>20 clinical trials	IIII/II II II/II	Recruiting	/
¹³¹ I-GMIB- Anti-HER2- VHH1	Precirix	HER2-positive breast cancer patients Advanced HER2-positive Breast, Gastric, GEJ Cancer	HER2	Targeted radionuclide therapy	NCT02683083 NCT04467515	I I/II	completed Recruiting	91 /
LDOS-47	Helix BioPharma	NSCLC NSCLC Lung adeno carcinoma Advanced pancreatic cancer	CEA CAM6	Immuno conjugate	NCT02340208 NCT02309892 NCT03891173 NCT04203641	I/II I II I/II	Completed Completed Terminated Recruiting	94 95 / /
LAVA-051	LAVA Therapeutics	Relapsed/ refractory CLL, MM and AML	CD1d	BiTE	NCT04887259	I/II	Recruiting	101
LAVA-1207	LAVA Therapeutics	Refractory metastatic prostate cancer	PSMA	BiTE	NCT05369000	I/II	Recruiting	102
SAR444200	Ablynx/Sanofi	Advanced solid tumor	GPC3	BiTE	NCT05450562	I/II	Recruiting	/
BCMA CAR-T Cilta-cel	Nanjing Legend Biotech Janssen	Relapsed/ refractory MM MM	BCMA	CAR T-cell	NCT03090659 NCT03548207 NCT04133636 NCT04181827 NCT04923893 NCT05257083 NCT05201781 NCT03881761	I/II Ib/II II III III III IV I	Completed Completed Recruiting Active Recruiting Recruiting Recruiting Recruiting	105 106–107 64–67 / / / / / /
CD19/20 Bispecific CAR-T Cells	Henan Cancer Hospital	B cell lymphoma	CD19 CD20	CAR T-cell	NCT03664661	I	Recruiting	/
BCMA CAR-T	Henan Cancer Hospital	Relapsed/ refractory MM	BCMA	CAR T-cell	NCT04323657	I/II	Completed	/
TC-110	TCR2 Therapeutics	Relapsed/ refractory NHL ALL	CD19	CAR T-cell	NCT04004637	I	Recruiting	/
CD7 CAR-T Cells	PersonGen BioTherapeutics	TLL, NK/T Cell Lymphoma, ALL	CD7	CAR T-cell	NCT02742727	I/II	Recruiting	/
CD7 CAR-NK cells	PersonGen BioTherapeutics	Relapsed/refractory Leukemia and Lymphoma.	CD7	CAR NK-cell	NCT04323657	I/II	Completed	/
TC-110	TCR2 Therapeutics	Relapsed/ refractory NHL ALL	CD19	CAR T-cell	NCT03907852	I/II	Recruiting	/
Gavo-cel	TCR2 Therapeutics	Advanced MSLN+ solid tumors	MSLN	CAR T-cell	NCT05451849	I/II	Recruiting	/
TC-510	TCR2 Therapeutics	Advanced MSLN+ solid tumors	MSLN	CAR T-cell	NCT05373147 NCT04503980 NCT04489862 NCT05089266	I	Recruiting	/

ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; BiTE: Bispecific T-cell engager; CLL: Chronic lymphocytic leukemia; GEJ: Gastroesophageal Junction; MM: Multiple myeloma; MoA: Mode of action; NK: Natural Killer; MSLN: Mesothelin; NHL: Non-Hodgkin lymphoma; PSMA: prostate-specific membrane antigen; TLL: T-lymphoblastic Lymphoma.

into the safety/tolerability profile of the drug, it was observed that most patients in both phase I studies showed drug-related adverse events, with some patients requiring exclusion from the studies. Despite these adverse events, the safety profile of BI 836,880 was determined to be manageable. In addition, another phase I study (NCT03972150) has been completed where BI 836,880 was tested in combination with an anti-PD-1 antibody in patients with advanced solid tumors [83]. Here, BI 836,880 shows similar safety and tolerability results compared to previous phase I studies. Currently, BI 836,880 is further tested in other phase I and II clinical trials to investigate its safety and efficacy as a combination therapy.

Envafolelimab, previously known as KN035, is a humanized anti-PD-L1 Nb fused with a silent human IgG1 Fc fragment, showing no ADCC and complement-dependent cytotoxicity to reduce adverse events. Binding of KN035 blocks PD-1/PD-L1 and CD80/PD-L1 signaling pathways, resulting in the promotion of T-cell activation [84]. Furthermore, preclinical studies showed that Envafolelimab displayed better T-cell activation and tumor-inhibitory effects compared to clinically approved Durvalumab [85]. In 2016, a first-in-human phase I study (NCT02827968) was performed whereby the safety and feasibility of subcutaneous (s.c.) Envafolelimab administration for the treatment of advanced, refractory solid tumors was assessed [86]. No dose-limiting toxicity was observed, and the most common adverse events included fatigue, nausea, and diarrhea. S.c. injections of Envafolelimab also displayed similar pharmacokinetic profiles compared to other mAbs and showed that a weekly injection would be sufficient for potential therapeutic effects. Based on these results, a phase II study was initiated in China (NCT03667170) to determine the efficacy and safety of Envafolelimab, whereby patients with locally advanced or metastatic mismatched repair deficient (dMMR) or microsatellite instability-high (MSI-H) solid tumors were treated with Envafolelimab monotherapy [68]. Interestingly, the efficacy of Envafolelimab was comparable to the reported phase II results of i.v. administered anti-PD-1 mAbs Nivolumab and Pembrolizumab in similar cancer patient subjects. Furthermore, Envafolelimab treatment had an acceptable safety profile with adverse effects such as decreased neutrophil and white blood cell counts, rash, and asthenia, which had also been reported for other PD-1/PD-L1 inhibitors. Based on these results, the s.c. treatment of MSI-H or dMMR advanced solid tumors with Envafolelimab has been conditionally approved in China [87]. Furthermore, two additional phase I studies in China (NCT03101488) and Japan (NCT03248843) have been reported with similar results [84]. Currently, several additional clinical studies are ongoing to test Envafolelimab, including combination therapies, which we have not included in Table 3.

Besides PD-L1 blockade using inhibitory Nbs, a humanized anti-CTLA-4 Nb fused to an Fc tail (KN044) is currently being tested in a phase I study of advanced solid tumors via i.v. injection (NCT04126590). However, further preclinical details about the Nb-based inhibitor are not in the public domain. Finally, a humanized bispecific Nb, targeting both PD-L1 and CTLA-4, fused to an Fc tail (KN046) has also been developed.

To date, this Nb-based immune checkpoint inhibitor is being tested in multiple phase II and phase II/III clinical trials for multiple oncological disorders, including NSCLC, pancreatic cancer, and hepatocellular carcinoma. However, to the best of our knowledge, no peer-reviewed reports have been published about these results.

3.2. Nanobody-based targeting agents in oncology trials

Several mAbs have already been clinically approved for targeted radioimmunotherapy and as antibody-drug conjugates in the clinic. However, Nbs have gained a lot of interest as alternative targeting agents, due to their small size and good tissue penetration. On a preclinical level, multiple studies have already shown the feasibility of using Nbs, conjugated to several types of cytotoxic molecules, as anti-cancer agents [59]. Based on these, two targeted Nb-based therapies are currently being tested clinically: a Nb-directed radionuclide therapy and a Nb-directed enzyme prodrug therapy [88,89].

As described above, labeling of Nbs with diagnostic radionuclides allows the noninvasive imaging of tumor or tumor-associated cells. However, Nbs can also be labeled with therapeutic radionuclides such as the β -emitters ^{131}I or ^{177}Lu , which upon binding to antigen-expressing cancer cells can induce DNA damage and induce anti-cancer immune responses resulting in subsequent killing of these cells. In 2017, our research team reported the preclinical use of an ^{131}I -labeled anti-HER2 Nb (^{131}I -GMIB-Anti-HER2-VHH1) as therapeutic in mice with HER2-positive tumor xenografts [90]. The ^{131}I -labeled anti-HER2 Nb showed similar fast and specific tumor retention properties as the ^{68}Ga -labeled variant that was described in section 2.1. Importantly, in contrast to radio-metal-labeled Nbs (e.g. labeled with ^{68}Ga or ^{177}Lu via chelation) ^{131}I -GMIB-Nbs are less retained in the kidneys upon renal filtration, and the absorbed dose in the tumor was higher than in the kidneys. Treatment of HER2-positive tumor-bearing mice resulted in a longer median survival, increased absence of tumors after treatment, and no signs of toxicity or mortality. Based on these results, a first-in-human phase I study (NCT02683083) was performed whereby six healthy individuals were included to assess safety, biodistribution, and dosimetry of the ^{131}I -GMIB-Anti-HER2-VHH1. No drug-related adverse events and a favorable biodistribution were observed, with the kidney being the primary organ for Nb excretion. In addition, uptake of ^{131}I -GMIB-Anti-HER2-VHH1 was observed in lesions in three patients with HER2-positive breast tumors during the phase I clinical trial [91]. Based on these results, a phase I/II dose escalation and expansion clinical trial (NCT04467515) is currently ongoing to further test the safety, dosimetry, tolerability, and efficacy of ^{131}I -GMIB-Anti-HER2-VHH1 in patients with HER2-positive tumors.

Currently, a Nb-directed enzyme prodrug therapy (L-DOS47) is also being clinically tested as a potential treatment for NSCLC patients. L-DOS47 is an immunoconjugate whereby a Nb targeting CEACAM6, an antigen overexpressed in several cancers including lung cancer, is fused with an urease moiety that converts endogenous urea to ammonia, resulting in an increased pH in the TME and induction of

tumor cytotoxicity [92,93]. Based on the promising preclinical results, a phase I/II dose escalation study was initiated where L-DOS47 was tested as a monotherapy in NSCLC patients (NCT02340208). L-DOS47 treatment was safe and tolerable [94]. While these data have been presented at conferences, no results have been published in a peer-reviewed journal. However, L-DOS47 has further been tested in different clinical trials as a combination therapy. This includes a phase I study (NCT02309892) where the safety and tolerability of L-DOS47 in combination with pemetrexed and carboplatin were assessed in NSCLC patients [95]. In total, 14 patients received L-DOS47 in combination with the chemotherapeutic agents. All patients showed adverse events, of which 12 had at least one event that was graded higher or equal to three on the severity scale (e.g. adverse events which are at least severe or medically significant but not immediately life-threatening). This included decreased neutrophil or white blood cell count. Three patients had adverse events that led to discontinuation, of which one case involved pneumonitis, which was possibly related to L-DOS47. Furthermore, all patients showed a non-dose-dependent immunogenic response to L-DOS47. Although the study was not designed to look into efficacy, treated patients showed promising overall response rates and clinical benefit rates. L-DOS47 has also been tested in a phase II study (NCT03891173) to evaluate its safety and tolerability in combination with vinorelbine and cisplatin in lung adenocarcinoma patients. However, this study was terminated for budgetary reasons. Currently, L-DOS47 is also being tested in a phase I/II study in advanced pancreatic cancer patients in combination with doxorubicin (NCT04203641).

Besides redirecting toxic agents to cancer cells, Nbs are also clinically employed as targeting agents, for BiTEs and CAR T-cells, to redirect and activate T-cells [62]. Here, Nbs are also being investigated as alternatives of scFv fragments due to their innate stability and other biophysical advantages [62]. On the one hand, BiTEs comprise two targeting domains with one domain (usually) targeting part of the T-cell receptor (TCR) (CD3) and another domain targeting a tumor-specific antigen. To date, one BiTE (Blinatumomab) consisting of two scFvs, targeting CD3 and CD19, has already been approved for the treatment of acute lymphoblastic leukemia (ALL) [96,97]. In parallel, three Nb-based BiTEs are currently being clinically investigated: LAVA-051, LAVA-1207, and SAR444200. On the other hand, Nb-based CAR T-cells, also known as nanoCAR-T cells, are also widely investigated [98]. Currently, already one nanoCAR-T cell therapy (Ciltacabtagene autoleucel/Cilta-cel) has been clinically approved, while multiple other nanoCAR-T cells are being investigated in clinical trials [99].

LAVA-051 is a bispecific Nb targeting both the CD1d and the V δ 2-TCR chain of V γ 9V δ 2-T-cells [100]. CD1d is a glycoprotein expressed in chronic lymphocytic leukemia (CLL), MM cells, and acute myeloid leukemia (AML) cancer cells. Binding of LAVA-051 results in anti-tumoral effects to CD1d+ tumors *in vitro* and *in vivo* via activation of both V γ 9V δ 2-T-cells and type 1 NKT cells [100]. Based on these results, a multicenter, open-label phase I/IIa study (NCT04887259) is ongoing to evaluate the safety, tolerability, and preliminary efficacy of LAVA-051 in CLL, MM, and AML patients. While the

study is still ongoing, preliminary results show that LAVA-051 is well tolerated and dose escalation is still ongoing based on the preliminary results [101].

LAVA-1207 is a humanized bispecific Nb-Fc fusion consisting of a Nb targeting the prostate-specific membrane antigen (PSMA) and the V δ 2-TCR chain. Preclinical results showed the ability of LAVA-1207 to induce killing of prostate cancer cells with an EC50 in the picomolar range [102]. Currently, a phase I/IIa first-in-human dose escalation study (NCT05369000) is ongoing to test the safety and tolerability of LAVA-1207 in refractory metastatic prostate cancer patients. Here, preliminary results also indicate that LAVA-1207 is well tolerated in early dose escalation, while future results will give more insight into the optimal dose [102].

SAR444200 is a BiTE consisting of a GPC3-targeting and a TCR-targeting Nb. GPC3 is a protein overexpressed on several solid tumors including hepatocellular carcinoma [103]. While not much is known about SAR444200, it is currently being tested in a phase I/II dose escalation and expansion study (NCT05450562) in advanced solid tumors.

Cilta-cel is the first nanoCAR-T cell therapy that has been FDA-approved as a treatment for relapsed/refractory MM patients [99]. Cilta-cel makes use of two Nbs targeting different epitopes of the B cell maturation antigen (BCMA), a protein that is highly expressed on the cell surface of malignant plasma cells [104]. In 2017, a multicentre phase I/II study (NCT03090659) was initiated to test the safety and efficacy in relapsed/refractory MM patients. Administration of Cilta-cel resulted in most patients experiencing adverse events and a cytokine release syndrome [105]. However, these adverse events were manageable in most patients, indicating a favorable safety profile. Interestingly, the administration of Cilta-cel showed an overall response rate of 88%, which was even observed over a period of 4 years after administration. This was higher compared to the overall response rate (73%) of Ide-cel, another approved BCMA-targeted CAR T-cell therapy. Based on these results, a phase Ib/II study (NCT03548207) was initiated to further study the efficacy of Cilta-cel. In 97 treated patients, an overall response rate of 97% was observed with a time to first response of 1 month [106,107]. Currently, an additional phase II study, where six different patient cohorts are treated with Cilta-cel in different clinical settings, has been initiated (NCT04133636). While the study is still ongoing, preliminary results allow similar conclusions as the previous clinical trials, indicating that Cilta-cel may be approved for additional MM subsets [64–67]. In addition, multiple phase III studies (NCT04181827, NCT04923893, and NCT05257083) are ongoing to compare the efficacy and safety of Cilta-cel to different standard therapies in different MM disease settings and even a phase IV study (NCT05201781) is ongoing to study the long-term safety. Besides Cilta-cel, four additional nanoCAR-T cell therapies and one nanoCAR-NK cell therapy for hematological disorders are currently in phase I clinical trials (NCT03881761, NCT03664661, NCT04323657, NCT04004637, and NCT02742727) (see Table 3). Besides the treatment of hematological diseases, nanoCAR-T cells are also clinically investigated as a treatment for solid tumors. Gavo-cel (also known as TC-210) is a nanoCAR-T cell therapy, using

a MSLN-targeting Nb as targeting moiety [108]. MSLN is expressed in several solid cancers, including mesothelioma, ovarian, lung, and pancreatic cancer, while only being weakly expressed in healthy mesothelial cells [109]. Preclinical studies showed potent antitumoral effects on MSLN-positive solid tumors *in vitro* and *in vivo* [108]. Interestingly, Gavo-cel showed fast kinetics with regard to tumor infiltration and activation *in vivo*, resulting in early tumor rejection. Based on these results, a phase I/II study (NCT03907852) was initiated to determine the recommended dose and to evaluate the efficacy of Gavo-cel in patients with advanced MSLN-expressing tumors. In parallel, two types of anti-MSLN nanoCAR-T cells, which also block the PD-1/PD-L1 interaction by either a PD-1-CD28 switch receptor (extracellular domain of PD-1 is fused to transmembrane and cytoplasmic domain of CD28) or secretion of soluble anti-PD-1 Nbs, are also being investigated in phase I or I/II clinical trials (NCT05451849, NCT05373147, NCT04503980, NCT04489862, and NCT05089266).

3.3. Preclinical developments of Nb-based therapeutics

While several Nb-based anti-cancer therapeutics are already clinically investigated, several novel therapeutic strategies, involving Nbs, are still being investigated preclinically and could make it into clinical trials in the near future. Primarily, efforts are being made to use Nbs as agents for targeted therapy and/or immuno-oncology purposes.

As mentioned in section 3.2, Nbs are currently already being clinically tested as targeting agents to redirect therapeutic radionuclides and enzymes toward tumors. However, preclinical studies have also investigated the potential of Nbs to redirect other toxic agents toward cancer cells, including toxins and even photosensitizers. Among the investigated toxins are pseudomonas exotoxin A, cucurmosin, and auristatins, and when conjugated to Nbs targeting different antigens (e.g. CD7, EGFR, GPC3, HER2, CD20, and MMR), enhanced tumor cytotoxicity was seen *in vitro* and/or *in vivo* [18,59,110,111]. While showing promise, the main limitations of these approaches are potential issues with safety and tolerability. Photodynamic therapy makes use of photosensitizers, typically IRDye700dx, that induce reactive oxygen species production upon activation with near-infrared light [112]. Conjugation of these photosensitizers to targeting agents, such as Nbs, allows for the specific accumulation of the photosensitizers in tumors. Next, local illumination of the tumor with near-infrared light will result in the accumulation of reactive oxygen species in the tumor and the specific killing of cancer cells. To date, multiple Nbs targeting cancer-specific antigens (e.g. EGFR, US28, c-MET, and HER2) have been conjugated to IRDye700dx and showed the ability to kill cancer cells *in vitro* and *in vivo* [113–117]. While this targeted therapy shows promise, it has the same limitations as image-guided surgery, in that it can only be applied to lesions lying maximally 1 cm deep in the tissue due to limited tissue penetration of the light source. However, since targeted photodynamic therapy with mAbs is already being used clinically, it is not unlikely for Nb-targeted photodynamic therapy to be tested clinically in the near future [118].

With respect to immuno-oncology, a plethora of preclinical studies using therapeutic Nbs have been published in the last decade. Here, Nbs have been used as immune checkpoint inhibitors targeting several immune checkpoints (e.g. CTLA-4, PD-1, PD-L1, and TIGIT), cancer vaccines, nanoCARs, immune cell engagers, immune cell-targeting agents, and agents to deliver cytokines. In this review, we highlight below an example of the latter type only, which could be translated into the clinic soon. More information on these different preclinical applications can be found in the review of Awad & Meeus et al. [62].

As cytokines play a key role in the TME, neutralization, or delivery of cytokines, depending on their effect on tumor growth, to the TME has been investigated. Currently, the use of therapeutically relevant doses of cytokines is hampered by systemic cytokine toxicity. Therefore, conjugation of mutant cytokines, with reduced affinity for their receptors, to Nbs to specifically deliver these cytokines to relevant cells in the TME can result in the successful eradication of tumors at lower cytokine doses [119]. For instance, the specific delivery of type I IFN to CD20^{pos} lymphomas or Clec9A^{pos} dendritic cells via conjugation to CD20- or Clec9A-targeting Nbs resulted in a strong antitumoral effect [120,121]. Furthermore, complete tumor regression was observed upon co-treatment with chemotherapy, immune checkpoint therapy, or low dose TNF. Likewise, conjugation of a mutant version of IL-1 β to a CD8-targeting Nb resulted in a cellular adjuvant that only showed a modest effect as monotherapy but enhanced adoptive T-cell transfer efficacy and synergized with other Nb-conjugated cytokine treatments [122].

4. Conclusions

mAb-based diagnostics and therapies have impacted different fields of oncology in the last decades. Despite this impact, the overall success of some of these applications is hampered due to the physical nature of conventional antibodies. As such, the use of Nbs has gained much attention in recent years. In this review, we highlight several Nb-based diagnostics and treatments that are currently in clinical trials or even approved, showing the success of Nbs in the field of oncology. Furthermore, novel Nb-based diagnostics and therapeutics are further being developed and are likely to evolve toward clinical translation in the near future. Overall, these studies indicate the value of Nbs in the field of oncology and show where Nbs can complement current cancer patient management.

5. Expert opinion

Since the initial discovery of heavy chain-only antibodies in *camelidae* in 1993, Nbs have garnered quite some attention as novel research tools, diagnostics, and therapeutics [9]. While Nbs are sometimes branded as alternatives to mAbs, we would argue that Nbs and mAbs should not be seen as competition but rather complementary technologies. As described in Table 1, mAbs and Nbs have distinct characteristics, which makes them ideal for specific purposes. Three of

the main differing characteristics, which highlights the complementarity of these molecules are their size, paratope, and *in vivo* half-lifetime. In general, Nbs could be more suited than mAbs for targeting complex transmembrane proteins, such as G protein-coupled receptors and channels, as they can fit more easily in binding pockets due to their small size and convex paratope [123,124]. To date, clinical translation of therapeutic compounds targeting these types of proteins is still underwhelming. As such, Nbs could fill this gap and it would not be surprising if more Nb-based therapeutics targeting complex transmembrane proteins would enter the clinic in the coming years. In contrast, the short *in vivo* half-lifetime of Nbs makes them less suited for a long-lasting therapeutic effect compared to mAbs. While their *in vivo* half-lifetime can be extended (see paragraph 1), mAbs are inherently more suited to induce a long lasting functional effect *in vivo*. However, a short *in vivo* half-life time is sometimes an advantage for purposes such as targeted delivery of radionuclides or toxic agents, whereby Nbs could be more suited. Despite the generally shorter *in vivo* half-lifetime, it is important to note that it is becoming more clear that the Nb format (monovalent vs multivalent) can have a big influence on the half-lifetime (due to changes in size or albumin binding) and tumor penetration (due to changes in size and avidity) and should be taken into account when developing a Nb-based diagnostic or therapeutic [125]. It is important to note that Nbs can be seen as tools that can be functionalized based on the needs for diagnosis and/or therapy, which does not necessarily apply to mAbs. Overall, it is clear that Nbs and mAbs serve complementary purposes and have to be chosen on an individual basis as targeting compounds.

After the initial discovery, multiple patents were filed on the generation and therapeutic use of Nbs. Based on this intellectual property, a spin-off company called Ablynx, which had exclusive rights to these patents, was set up. In 2007, Ablynx initiated the first clinical trials with a Nb, which would later be approved and rebranded as Caplacizumab. However, due to these patents, only a limited number of clinical trials were initially started. As of 2016, these patents have expired. This, in combination with an increase in preclinical developments with Nbs, has resulted in a steady increase of initiated clinical trials in the last 7 years including oncological clinical trials (see Tables 2 and 3). It seems only logical that this increase in oncological clinical trials with Nbs will become even bigger over the coming years as more and more preclinical studies are being published on a yearly basis (Figure 4).

It is remarkable that, although the field of disease diagnosis is economically less interesting than the therapeutic application field, quite some clinical translation in the field of Nb-based diagnostic imaging in oncology has already happened. The main underlying reason behind this is the less strict requirements to initiate diagnostic clinical trials as compared to therapeutic clinical trials. Quite some of the strict rules, set for pharmaceuticals, do not apply for radiopharmaceuticals and are, as such, considered as a special group of medicines. Indeed, adaptations have been made regarding micro-dosing, single dosing, and the use of short-lived radiopharmaceuticals, which has enabled easier clinical translation of Nb-based diagnostic tracers. Moreover, the cost for clinical translation is

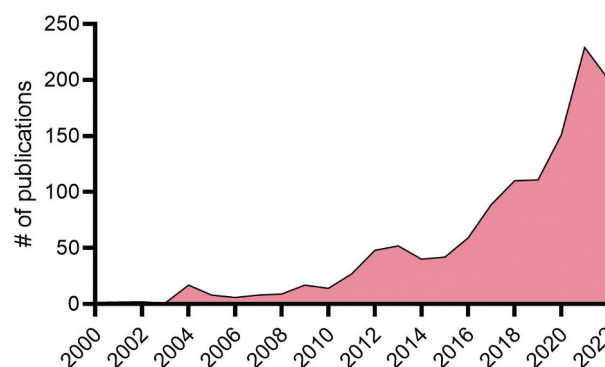


Figure 4. Overview of published studies on nanobodies in oncological research. The number of papers were determined via PubMed search using terms 'nanobody' and 'cancer'.

significantly lower due to lower mass doses needed for radio-tracers, lower toxicology demands, less repeated injections, and less costs involving the follow-up of patients. These parameters, combined with the characteristics of Nbs, let us believe that in the coming years more researchers will push even more Nb-based diagnostic imaging toward the clinic.

A major drawback in Nb-based diagnosis and therapy is the limitation of targetable proteins. Due to their inability to cross cell membranes, Nbs (but also mAbs) can only be employed against extracellularly expressed targets. However, as 95% of tumor antigens are intracellular, some research is now focused into raising Nbs (and mAbs) toward these intracellular tumor antigens whereby they are presented as peptides on MHC-peptide complexes on the tumor surface [126]. These so-called TCR-like nanobodies could open up a new plethora of targetable proteins for Nb-based targeted therapies. An example of this is the TCR-like nanobody GPA7, recognizing gp100 209–217/HLA-A2, which has successfully been integrated in a nanoCAR-T approach to kill melanoma cells [127]. While TCR-like mAbs have also been developed, Nbs could be seen as a complementary (or even better) approach as it is not unlikely that Nbs could bind in different (or even better) ways to the concave epitopes of the MHC-peptide complexes.

Finally, one question which is essential for the clinical translation of Nbs, and still remains a point of investigation is the immunogenicity of Nbs. To date, it is assumed that Nbs are generally non-immunogenic due to their small size, fast half-life time, and high homology with the human IGHV3 family [15,128]. However, Nbs are derived from non-human mAbs and can still induce immunogenic responses, especially when administered repeatedly and at high doses. With the increasing number of clinical trials, more and more data are becoming available to give insight into the general immunogenicity profile of Nbs. Indeed, it seems that Nbs overall show low immunogenicity profiles with no or a small number of patients developing anti-drug antibodies during Nb treatment [129]. However, cases have been reported where Nbs are highly immunogenic, resulting in the termination of the study [75,130]. Humanization is one of the strategies that has been employed to lower potential immunogenicity and there are currently already multiple strategies to humanize Nbs [129]. However, the question remains whether humanization is the best approach to render Nbs non-

immunogenic, as the reported immunogenic Nbs were humanized, while other non-humanized Nbs do not show any immunogenicity [15]. Furthermore, humanization of Nbs will result in changes in the different FR regions of these Nbs. As these FR regions are crucial for the structural folding, Nb stability, and sometimes even epitope binding, humanization of Nbs can lead to detrimental changes including loss of binding, increased protein instability, and/or protein aggregation. As such, humanization of Nbs needs to be performed on an individual basis and humanized Nbs need to be validated thoroughly. While it remains likely that immunogenicity of Nbs needs to be handled individually, the increased number of phase I and II data with Nbs will give more insight into the immunogenicity of Nbs.

Abbreviations

IgG	Immunoglobulin G
mAbs	Monoclonal antibodies
kDa	Kilodalton
scFv	Single chain variable fragments
Fabs	Antigen binding fragments
Nbs/VHH	Nanobodies
FR	Framework region
CDR	Complementarity determining region
nM	nanomolar
pM	picomolar
V _H	Variable domain of the heavy chain
V _L	Variable domain of the light chain
Fc	Fragment crystallizable
CT	Computed tomography
MRI	Magnetic resonance imaging
¹⁸ F	Fluor-18
FDG	Fluorodeoxyglucose
PET	Positron emission tomography
SPECT	Single photon emission computed tomography
⁶⁸ Ga	Gallium-68
^{99m} Tc	Technetium-99 m
mSv	millisievert
HER2	Human epidermal growth factor receptor 2
CLDN18.2	Claudin 18.2
CD	Cluster of differentiation
MMR	Macrophage mannose receptor
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PD-1	Programmed cell death protein 1
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CAR-T cells	Chimeric antigen receptor – T cells
TIGIT	T cell immunoreceptor with Ig and ITIM domains
LAG-3	Lymphocyte activation gene 3
NSCLC	Non-small cell lung cancer
⁸⁹ Zr	Zirconium-89
TAM	Tumor associated macrophage
TME	Tumor microenvironment
EGFR	Epidermal growth factor receptor
CEA	Carcinoembryonic antigen
HER3	Human epidermal growth factor receptor 3
MSLN	Mesothelin
PSMA	Prostate specific membrane antigen
MHC-II	Major histocompatibility complex II

SIRPα	Signal regulatory protein α
CAIX	Carbonic Anhydrase IX
NHS	N-Hydroxysuccinimide
MoA	Mode of action
BiTe	Bispecific T-cell engager
nanoCAR-T	Nanobody based CAR-T cells
VEGFR	Vascular endothelial growth factor
c-Met	Tyrosine-protein kinase Met
CEACAM6	Carcinoembryonic antigen related cell adhesion molecule 6
CXCR4	C-X-C chemokine receptor type 4
ACKR3	Atypical chemokine receptor 3
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
IL-23	Interleukin-23
TNF-α	Tumor necrosis factor- alfa
CXCL12	C-X-C chemokine receptor type 12
MM	Multiple myeloma
DR5TRAIL	Death receptor 5 Tumor necrosis factor-related apoptosis-inducing ligand
i.v.	intravenous
ADCC	Antibody-dependent Cellular Toxicity
Ang-2	Angiopoietin-2
s.c.	subcutaneous
dMMR	Deficient mismatch repair
MSI-H	microsatellite instability-High
¹³¹ I	Iodine-131
¹⁷⁷ Lu	Lutetium-177
DNA	Deoxyribonucleic acid
TCR	T cell receptor
ALL	Acute lymphoblastic leukaemia
CLL	Chronic lymphocyte leukaemia
AML	Acute myeloid leukaemia
NKT	Natural killer T cells
EC50	Effective concentration 50
GPC3	Glypican-3
BCMA	B-cell maturation antigen
IFN	Interferon
Clec9A+	C-Type Lectin Domain Containing 9A
IL-1β	Interleukin 1 beta

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Declaration of interest

N. DeVoogdt is co-founder and scientific consultant at Precirix NV and ABSCINT NV. J. Van Ginderachter and G. Raes are co-founders of ABSCINT NV. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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