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LEADING ARTICLE

Targeting Cytotoxic T-Cell Effector Function with Granzyme B PET: Translational Advances Toward Predicting Immunotherapy Outcomes

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Abstract

The clinical success of cancer immunotherapy has highlighted the urgent need for biomarkers capable of capturing dynamic and functional anti-tumor immune activity. Granzyme B (GzmB), a serine protease released by activated cytotoxic $CD8^+$ T lymphocytes and natural killer cells during target-cell killing, represents a proximal and mechanistically linked marker of immune effector function. Positron emission tomography (PET) tracers targeting GzmB enable non-invasive, whole-body assessment of cytotoxic activity in vivo, potentially overcoming the spatial and temporal limitations of tissue biopsy and conventional imaging. Preclinical investigations using ^{68}Ga- and ^{18}F-labeled peptide-based tracers, including [^{68}Ga]Ga-NOTA-GZP and [^{18}F]AlF-mNOTA-GZP, consistently demonstrate early discrimination between responders and non-responders to immune checkpoint blockade and combination therapies, often preceding measurable tumor shrinkage or metabolic changes on [^{18}F]FDG PET. Emerging first-in-human studies further support the feasibility, safety, and predictive potential of GzmB PET across multiple tumor types. Despite promising results, several challenges remain, including optimal imaging timing, signal heterogeneity related to non-canonical GzmB sources, and the need for standardized quantitative thresholds and multicenter validation. Overall, GzmB PET represents a biologically grounded and translationally advanced approach for early response assessment, patient stratification, and potential theranostic applications in precision immuno-oncology.

Key Points

- Granzyme B PET imaging measures active cytotoxic cell killing rather than immune-cell presence alone.
- This approach enables earlier prediction of immunotherapy response than tumor size or changes on PET with [18F]FDG.
- Different Granzyme B tracers offer distinct kinetic profiles and clinical logistics.
- Early clinical studies show promising diagnostic accuracy, despite small cohorts.
- Biological and kinetic factors complicate signal interpretation in some settings.
- Future clinical translation requires protocol standardization and multicenter validation.

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1 Introduction

Over the past decade, the rapid expansion of cancer immunotherapy has transformed treatment options for many patients with both solid tumors and hematologic malignancies [1]. At the same time, however, clinical responses remain highly heterogeneous, creating an urgent need for biomarkers that report, in space and in time, the immune mechanisms that determine therapeutic success or failure [2]. Conventional anatomic imaging with computed tomography (CT) and functional imaging such as magnetic resonance imaging (MRI) and fluorine-18 fluorodeoxyglucose positron emission tomography ([^{18}F]FDG PET) provide important measures of tumor burden and metabolism but are indirect with regard to the cellular immune processes – in particular the cytotoxic activity of effector lymphocytes—that underlie many durable responses to immunotherapy [3, 4]. While current imaging modalities provide valuable insights, their inherent limitations leave a critical gap in our ability to predict immunotherapy outcomes. Addressing this unmet clinical need requires a shift toward more functional markers; consequently, Granzyme B (GzmB) has emerged as a compelling target for specialized molecular imaging.

GzmB is a serine protease stored in granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells and is a principal mediator of granule-exocytosis–dependent killing [5]. Following antigen recognition and immunological synapse formation, CTLs and NK cells polarize lytic granules toward the target cell and release perforin and granzymes; perforin facilitates delivery of GzmB into the target cell cytosol, where GzmB cleaves substrates that trigger programmed cell death [6]. GzmB activates multiple apoptotic pathways, including direct and indirect activation of executioner caspases such as caspase-3 and caspase-7, as well as upstream initiator caspases such as caspase-9, ultimately converging on apoptotic cell death [7]. Production, activation, and release of GzmB are tightly regulated at transcriptional, translational and post-translational levels, and cytotoxic cells express endogenous inhibitors (e.g., Serine Protease Inhibitor 9 and its murine homolog Spi6) to limit self-injury [8]. These canonical features—regulated storage, stimulus-dependent release, and potent intracellular substrates—underlie the rationale for regarding GzmB as a proximate, mechanistic readout of lymphocyte-mediated tumor cell killing.

Importantly, however, recent work has expanded the functional repertoire of GzmB beyond the classical perforin-dependent apoptotic pathway. GzmB can be released into the extracellular milieu in enzymatically active or zymogen forms and can act in a perforin-independent manner on extracellular matrix (ECM) components and soluble matrix-associated factors, thereby promoting basement membrane remodeling, facilitating lymphocyte transmigration, and liberating pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF) from matrix stores [9]. Moreover, GzmB expression has been reported in a variety of non-classical cellular sources—including mast cells, plasmacytoid dendritic cells, regulatory T cells, and certain myeloid populations—where its activities can be contextually immunosuppressive, protumoral, or regulatory rather than strictly cytolytic [10]. Perforin-independent signaling and non-lethal GzmB-dependent modifications further complicate the interpretation of measurable GzmB activity in tissues. Collectively, these canonical and non-canonical roles argue that an in vivo GzmB signal can reflect multiple biologic processes related to anti-tumor immunity, immune regulation, and tissue remodeling [9]. Target selectivity in GzmB–directed peptide substrates is often suboptimal, but can be modulated through minimal sequence engineering, such as the IEFD (Ile-Glu-Phe-Asp)-to-IEPD (Ile-Glu-Pro-Asp) substitution, which has been shown to enhance discrimination over other granzymes and underpins the development of rationally designed imaging probes [11]. Nevertheless, absolute enzymatic specificity cannot be assumed, given the unresolved potential for cross-reactivity with caspase-dependent cleavage mechanisms.

Before the emergence of protease-targeted tracers, several molecular imaging strategies were developed to visualize different components of the antitumor immune response in vivo. These approaches include radiotracers targeting immune cell populations such as CD8-positive T lymphocytes [12, 13], immune checkpoint molecules [14, 15], and markers of T-cell activation [16, 17]. Although these strategies provide biologically relevant information regarding activated effector lymphocytes, they may not directly quantify cytolytic protease release or target-cell killing with the same specificity as GzmB-based tracers.

Together, these biological considerations define both the promise and the complexity of GzmB–targeted PET imaging. From a translational perspective, a radiolabeled probe capable of selectively reporting enzymatically active GzmB might offer a unique opportunity to capture a proximal and functional measure of cytotoxic effector activity, one that may precede and ultimately predict downstream anatomic or metabolic changes observed with conventional imaging. In this context, serial tumor biopsies remain the current reference standard for assessing therapy response at the tissue level; however, their clinical utility is limited by their invasiveness and by sampling bias, as well as by their inability to fully capture the spatial and temporal heterogeneity of the tumor microenvironment during dynamic immunomodulation.

Building on this premise, the present review traces the translational trajectory from mechanistic insight to in vivo application of GzmB-targeted imaging.

2 Preclinical Studies

Several radiotracers targeting GzmB have been developed and assessed in preclinical studies. Preclinical evaluation of GzmB-targeted PET tracers has predominantly been performed in syngeneic, immunocompetent murine tumor models, which are essential to preserve functional host immune responses and to enable accurate assessment of cytotoxic T-cell and NK-cell activity [18]. In contrast, immunodeficient xenograft models are not suitable for this class of imaging agents, as the absence of functional adaptive and innate immune effector cells [19] precludes the evaluation of GzmB–mediated cytotoxic activity, which represents the biological target of these tracers.

In 2017, Larimer et al. [11] were the first to design a PET imaging agent capable of detecting GzmB release from actively activated immune cells. This radiotracer was designed based on the optimal murine GzmB cleavage sequence, with additional modifications aimed at enabling reversible covalent interaction with the catalytic cysteine residue, as well as labeling with a suitable PET-detectable radiometal. Indeed, a short Gly-Gly-Gly linker was introduced to couple the peptide to the NOTA chelator, preserving inhibitory activity as confirmed by in vitro assays (Ki = 47±54 nM). The resulting conjugate (NOTA-GZP) was efficiently radiolabeled with gallium-68 (^{68}Ga) produced via a germanium-68/gallium-68 (^{68}Ge/^{68}Ga) generator system, having a physical half-life of 68 min, yielding high radiochemical purity (> 95%), favorable specific activity (5190 ± 1100 MBq/mg), and a half-life well matched to peptide pharmacokinetics, collectively supporting its suitability for in vivo PET imaging [11]. The tracer exhibited primary renal clearance, with significant physiological accumulation observed in the kidneys and bladder. This rapid pharmacokinetic profile enables repeated, non-invasive quantification of GzmB activity, facilitating its incorporation into clinical trial protocols and potentially reducing the need for serial biopsies. In line with these advantages, several studies [20–22] have examined ^{68}Ga-labeled radiotracers targeting GzmB, underscoring their potential utility for monitoring or early assessment of immunotherapy response. More recently, [^{68}Ga]Ga-grazytracer has emerged as a promising radiotracer, enabling sensitive and specific visualization of GzmB activity in vivo [23]. Its high target-to-background contrast supports its potential for early assessment of immunotherapy response and real-time monitoring of cytotoxic immune activation within tumors.

Subsequently, Zhao and co-workers [24] proposed a granzyme targeting Restricted Interaction Peptide specific to family member B (GRIP B), which was radiolabeled with copper-64 (^{64}Cu), as its approximately half-life ($t_{1/2}$ ≈ 13 h) permits imaging assessments across an extended post-injection timeframe, allowing determination of the most informative acquisition time point. The GRIP-B probe was designed using multiplex substrate profiling by mass spectrometry of recombinant human GzmB, identifying a preferred cleavage motif centered on the P2–P1 residues. On this basis, the sequence IEPDVSQV was selected for its validated specificity and favorable catalytic efficiency toward GzmB [24]. GRIP-B functions as a protease-activated switch probe in which GzmB-mediated cleavage removes a masking domain, restoring the amphipathic Temporin L helix and enabling membrane insertion. Upon activation, the cleaved radiolabeled fragment becomes anchored to nearby phospholipid membranes via the Temporin L–derived domain, effectively trapping the signal at the site of enzymatic activity. Consequently, at prolonged post-injection intervals (i.e., hours post-injection rather than seconds), the in vivo tissue distribution of the radiotracer serves as a surrogate measure of relative GzmB activity.

Subsequently, Goggi et al. introduced a fluorine-18–labeled agent, [^{18}F]AlF-mNOTA-GZP, featuring a longer half-life than ^{68}Ga (109.7 min) [25, 26]. The synthesis involved production of aqueous ^{18}F-fluoride via the [^{18}O(p,n)^{18}F] nuclear reaction, while the peptide scaffold NOTA–β-Ala–Gly–Gly–Ile–Glu–Phe–Asp–CHO (mNOTA-GZP) was assembled using standard fluorenylmethyloxycarbonyl chemistry and characterized by HPLC (high-performance liquid chromatography) and mass spectrometry [27].

The main results of the pre-clinical studies on the application of GzmB PET imaging are summarized in Table 1.

2.1 [^{68}Ga]Ga-NOTA Radiotracers

Across multiple preclinical and translational studies, [^{68}Ga]Ga-NOTA-GZP PET imaging has consistently emerged as a sensitive, non-invasive biomarker of effective antitumor immune activation. The first work regarding [^{68}Ga]Ga-NOTA-GZP was published in 2017 [11]. [^{68}Ga]Ga-NOTA-GZP should be classified as an activity-based imaging probe, as its signal generation depends on selective enzymatic cleavage by catalytically active GzmB, rather than passive substrate accumulation or binding. Importantly, a single amino acid substitution (IEFD to IEPD) was required to confer selectivity for human GzmB, highlighting species-specific substrate differences that must be considered in translational studies. The authors used CT26 tumor-bearing mice that showed different levels of granzyme and that were treated with anti-PDL1 and anti-CTLA4. This pilot experience demonstrated that GzmB PET can distinguish

Table 1 Preclinical studies of Granzyme B (GzmB) PET imaging

Author (year)	Radiotracer and mechanism	Model and therapy	Key findings (uptake and metrics)	Translational note
Larimer et al. (2017) [11]	[^{68}Ga]Ga-NOTA-GZP Irreversible peptide inhibitor	Balb/c, CT26; ICI combinations	TBR: Ctrl 0.96 vs. Comb 1.83. TR vs. NR classification.	Strong potential for early prediction of immunotherapy response.
Larimer et al. (2019) [20]	[^{68}Ga]Ga-NOTA-GZP Irreversible peptide inhibitor	Balb/c, CT26; C57BL/6, MC38; ICI combinations	TBR (MC38): Ctrl 1.37 vs. Comb 2.17. High utility in treatment selection.	Supports optimization of ICI regimens and treatment selection.
LaSalle et al. (2020) [21]	[^{68}Ga]Ga-NOTA-GZP Irreversible peptide inhibitor	Balb/c, CT26; C57BL/6, MC38; ICI combinations	Longitudinal TBR: Ctrl 1.62 vs. Comb 2.82 (12d). Correlates with immune activation.	Allows longitudinal monitoring of immune activation dynamics.
Goggi et al. (2020) [25]	[^{18}F]AlF-mNOTA-GZP GZMB targeting probe	Balb/c, CT26; C57BL/6, MC38; ICI combinations	%ID/g: Ctrl 0.23 vs. Comb 0.59 (CT26). TR vs. NR stratification.	Enables robust response stratification across diverse ICIs.
Goggi et al. (2020) [27]	[^{68}Ga]Ga-mNOTA-GZP Irreversible peptide inhibitor	Balb/c, CT26-WT; ICI combinations	%ID/g: Ctrl 0.25 vs. TR-Comb 0.60. Superior to conventional metabolic imaging.	Provides superior functional assessment compared with FDG PET.
Goggi et al. (2021) [26]	[^{18}F]AlF-mNOTA-GZP GZMB targeting probe	C57/BL6, HEPA 1-6; ICI combinations	%ID/g: Ctrl 0.35 vs. TR-Comb 0.93. NK-dominant response monitoring.	Applicable in immunosuppressive tumors with non-T-cell responses.
Goggi et al. (2021) [31]	[^{18}F]AlF-mNOTA-GZP GZMB targeting probe	Balb/c, CT26; ICI ± Chemotherapy	%ID/g: Ctrl 0.31 vs. TR-Comb 0.68. Assessment of cytotoxicity.	Assesses combined chemotherapy and immunotherapy cytotoxicity.
Zhao et al. (2021) [24]	[^{64}Cu]Cu-DOTA-GRIP B Proteolytically activated probe	Balb/c/C57BL6, CT26, MC38, EMT6; ICI	Kinetic modeling (k3, k4); TBR increases over time. Direct GZMB activity measure.	Introduces activity-based PET for direct immune effector monitoring.
de Aguiar Ferreira (2022) [33]	[^{68}Ga]Ga-NOTA-GZP [90Y]Y-GZP Theranostic pair	Balb/c, CT26; C57BL6, MC38; ICI ± 90Y therapy	TBR: Ctrl 0.7 vs. ICI 4.1. Established theranostic platform.	Establishes a theranostic platform combining imaging and RNT.
Hartimath et al. (2022) [32]	[^{18}F]F-AlF-mNOTA-GZP GZMB targeting probe	Balb/c, CT26; ICI ± CpG-ODN	%ID/g: Ctrl 0.20 vs. ICI+CpG 0.95. Stratification of vaccine-ICI response.	Evaluates combination therapies (CpG vaccine + ICI).
Jiang et al. (2023) [29]	[^{68}Ga]Ga-NOTA-GZP Irreversible peptide inhibitor	B16F10, MCF-7; [131I]-KN046	TNR Peak: 11.34 at 72h. Integration of RNT and real-time monitoring.	Supports integration of radionuclide therapy and immunotherapy.
Zhang et al. (2024) [22]	[^{68}Ga]Ga-NOTA-GZP Irreversible peptide inhibitor	BALB/c 4T1; C57BL/6 LLC; RT ± ICI	TBR: TR 1.53 vs. NR 1.13 (4T1). Prediction of abscopal effects.	Enables prediction of abscopal effects and timing optimization.

GZP β-Ala-Gly-Gly-Ile-Glu-Phe-Asp-CHO, *GRIP B* Granzyme targeting Restricted Interaction Peptide specific to family member B, *GZMB* granzyme B, *ICI* immune checkpoint inhibitor, *s.c.* subcutaneous, *p.i.* post-injection, *p.t.i.* post-tumor inoculation, *TR* tumor responder, *NR* non-responder, *TBR* target-to-blood-pool ratio, *TNR* target-to-normal ratio, *Ctrl* controls, *Comb* combination therapies

immunotherapy responders from non-responders prior to measurable changes in tumor volume, correlating strongly with cytotoxic T-cell activity and offering advantages over conventional biomarkers and [^{18}F]FDG PET, also in human melanoma samples.

Goggi et al. [27] performed comparative imaging studies and confirmed that among multiple radiopharmaceuticals probing immune biology, labeled with ^{68}Ga or ^{18}F, [^{68}Ga]Ga-NOTA-GZP showed the strongest correlation with CD8^{+} T-cell activity and treatment response, thus confirming the result by Larimer et al. [11], in colon-rectal tumor. After 2 years, the same group of researchers [20] demonstrated that quantitative GZP uptake robustly predicts response across tumor models (i.e., CT26/immunologically "cold" and MC38/immunologically "hot"), checkpoint inhibitor combinations (such as anti-PD-1 plus anti-CTLA-4), and by considering different dosing schedules. These results supported the use of [^{68}Ga]Ga-NOTA-GZP as an "early" efficacy biomarker for therapeutic optimization and clinical trial design, with assessment during the first cycles of immune checkpoint inhibition (typically one to two treatment cycles), thereby preceding conventional radiological response evaluation, which is generally performed at 6–8 weeks according to standard clinical trial protocols [28]. LaSalle et al. [21] demonstrated that GzmB PET reflects functional immune activation within distinct tumor microenvironments and can help identify pathways associated with response and resistance. The authors followed for a long time the activation of the immune systems in diverse tumor models, both CT26 and MC38, showing that the activation of T-lymphocyte was consistently different in the time and in accordance with the type of treatments.

More recent works extended its application to combination strategies, respectively in breast cancer and in melanoma [22, 29], demonstrating that [^{68}Ga]Ga-NOTA-GZP PET can non-invasively detect abscopal immune activation and predict response following radiotherapy plus checkpoint blockade [22]. Moreover, the agent can be applied as a pharmacodynamic tool to monitor enhanced immune activation in radionuclide-immunotherapy combinations, for example with ^{131}I, further supporting its translational value as a functional imaging biomarker of anti-tumor immunity [29].

2.2 [^{18}F]AlF-mNOTA Radiotracers

To address the logistical and physical limitations of ^{68}Ga-labeled tracers, such as dependence on on-site generators and higher positron energy that can degrade spatial resolution, research has shifted toward ^{18}F-labeled radiopharmaceuticals [30]. These offer superior imaging characteristics and facilitate large-scale distribution. A significant development in this field is [^{18}F]AlF-mNOTA-GZP, a peptide-based probe specifically engineered to target GzmB.

Goggi et al. [25] investigated the in vivo performance of [^{18}F]AlF-mNOTA-GZP using small-animal PET imaging to monitor response to immunotherapy in murine syngeneic colorectal cancer models, specifically the CT26 and MC38 tumors. Radiolabeling of mNOTA-GZP was performed using the Al[^{18}F]F-labeling approach. Briefly, aqueous [^{18}F] fluoride was first trapped on a QMA cartridge, eluted with saline, and reacted with $AlCl_3$ to form the [^{18}F]AlF intermediate. The reaction was then carried out with mNOTA-GZP at 100 °C for 15 min, followed by purification using carbon-18 solid-phase extraction to afford [^{18}F]AlF-mNOTA-GZP with high radiochemical purity (98–99%) [17]. The imaging readout clearly differentiated responding tumors from non-responders. In the CT26 model, control and non-responding tumors showed low uptake, whereas responding tumors treated with checkpoint inhibitors exhibited higher uptake. In contrast, MC38 tumors demonstrated higher baseline uptake, with responding tumors showing a further increase, particularly following combination therapy. Importantly, PET-derived tracer uptake correlated with immune activation assessed ex vivo. In CT26 tumors, uptake showed a strong correlation with CD8^{+} GzmB–positive T cells, while in MC38 tumors the correlation remained significant but more moderate.

In a subsequent study from the same group [31], GzmB PET with [^{18}F]AlF-mNOTA-GZP was used to assess response to oxaliplatin, 5-fluorouracil, anti-PD-1 therapy, and their combinations in CT26 syngeneic colon tumors. Quantitatively, [^{18}F]AlF-mNOTA-GZP tumor uptake was low in treated non-responders and consistently higher in responders. Tracer uptake significantly correlated with tumor growth inhibition. Correlative flow cytometry and unsupervised clustering demonstrated that increased PET signal reflected elevated levels of GzmB–expressing immune effectors, including CD8^{+} T cells and NK cells, with chemo-immunotherapy combination showing additive effects consistent with recruitment of distinct cytotoxic populations. Despite recognizing translational limitations—such as low absolute tumor uptake, potential off-target cleavage by caspases, and tumor heterogeneity—the authors concluded that GzmB PET could serve as a noninvasive marker of effective immune-mediated tumor cell killing in chemo-immunotherapy regimens, provided that sensitivity and background issues are addressed.

[^{18}F]AlF-mNOTA-GZP was also evaluated as a PET biomarker for stratifying response to immune checkpoint blockade in a syngeneic hepatocellular carcinoma model (HEPA 1-6) [26]. Mice bearing subcutaneous HEPA 1-6 tumors received intravenous tracer injection followed by PET/CT imaging approximately 1 h post-injection. Animals were treated with control IgG, anti-PD-1, anti-CTLA-4, or combination therapy, and tumor growth was monitored over time. Responding tumors showed consistently higher tracer

uptake than non-responders and controls, correlating with therapeutic efficacy. Notably, uptake of [^{18}F]AlF-mNOTA-GZP was more strongly associated with GzmB-positive NK cells than with CD8$^+$ T cells and correlated with increased intratumoral NK activity.

The same group further evaluated the performance of GzmB PET imaging with [^{18}F]AlF-mNOTA-GZP for monitoring response to an in situ vaccine (CpG-ODN 1585) combined with anti-PD-1 therapy in the murine CT26 colon cancer model [32]. Imaging was performed during the course of therapy following intravenous tracer administration, with PET acquisition at a standardized post-injection time point. GzmB PET effectively distinguished responders from non-responders across treatment groups. Responding tumors exhibited markedly higher tracer uptake compared to controls and non-responders, with the strongest signal observed in the group receiving intratumoral CpG-ODN and anti-PD-1 (Fig. 1). Notably, intratumoral delivery of the vaccine proved more effective than its systemic administration. These imaging findings were supported by flow cytometry analysis, which demonstrated a strong positive correlation between tracer uptake and the infiltration of GzmB-expressing CD8$^+$ T cells within the tumor microenvironment.

2.3 Preclinical Theranostic Applications

Targeted radionuclide therapy (TRT), by selectively accumulating in tumors via targeting ligands, induces tumoricidal effects through DNA damage and can create a pro-inflammatory microenvironment, showing potential synergy with ICIs. De Aguiar Ferreira et al. [33] explored a GZP as a theranostic agent: [^{68}Ga]Ga-GZP for PET imaging and [^{90}Y]Y-GZP for TRT, evaluated in combination with ICIs in murine colon cancer models. Two syngeneic models were investigated, including MC38 and CT26 tumors, representing distinct levels of immunogenicity. The ICI regimen (anti–PD-1 plus anti–CTLA-4) was given on days 3, 6, and 9, with imaging 3 days after the final dose. For therapy, low-dose (2.22 MBq) and high-dose (22.2 MBq) [^{90}Y]Y-GZP were administered after ICI treatment. Tumor volumes, overall survival and toxicity were assessed. PET/MR showed high [^{68}Ga]Ga-GZP uptake in kidneys and bladder, indicating renal clearance, with the highest non-renal uptake in liver. Tumor uptake increased in animals treated with immune-checkpoint inhibitors in both models, with significant differences. Blood pool uptake was similar across groups, while tumor-to-blood and tumor-to-muscle ratios were markedly higher after immunotherapy. MC38 tumors showed higher uptake than CT26, consistent with greater immunogenicity and responsiveness.

Therapeutically, high-dose [^{90}Y]Y-GZP after immunotherapy achieved complete tumor responses in both models. Survival improved significantly in high-dose groups, with median survival not reached in some cohorts. Immunological analyses further indicated that TRT did not impair immune cell infiltration: in some cases, CD4$^+$ and CD8$^+$ T-cell signals were preserved or increased. GzmB expression remained elevated following combination treatment, supporting sustained cytotoxic activity within the tumor microenvironment. Overall, these findings highlighted the dual role of GzmB–targeted agents as both pharmacodynamic biomarkers and potential platforms for image-guided radionuclide therapy, while emphasizing the

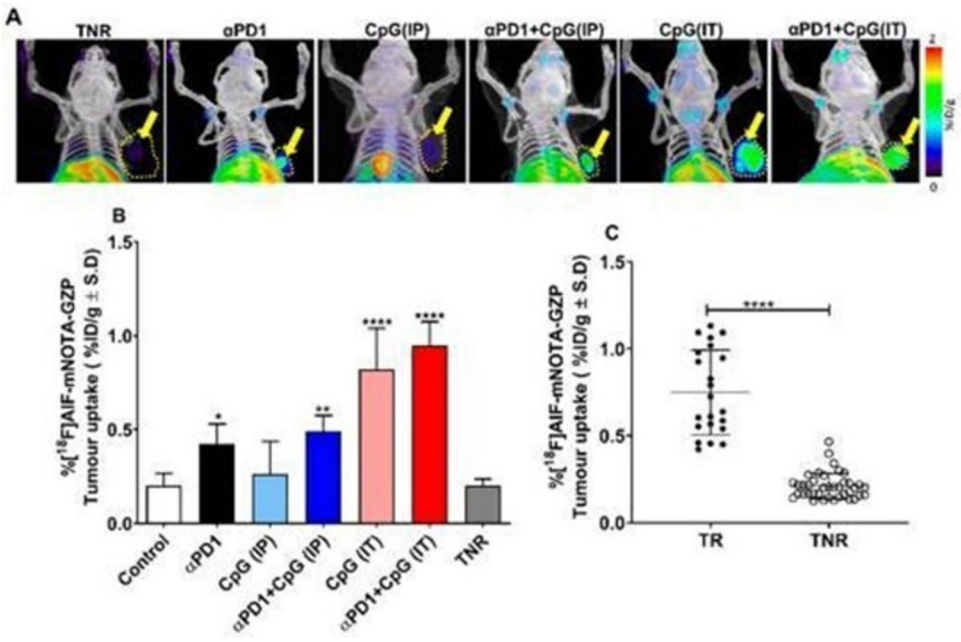

Fig. 1 **A** Fused [^{18}F]-AlF-mNOTA-GZP PET/CT reveals significantly higher tumor uptake in responders (TR) vs. non-responders (TNR), most prominently in intratumoral and aPD1+CpG combination groups. **B**, **C** Quantitative data (mean ± SD) 2 days post-treatment confirms these marked increases, effectively differentiating therapeutic outcomes. Reprinted from [32], under a creative commons attribution 4.0 international license (http://creativecommons.Org/licenses/by/4.0/). No changes were made

need for further optimization of dosimetry and safety prior to clinical translation.

3 Clinical Studies

Zhou et al. conducted one of the first studies aimed at enabling the clinical translation of GzmB-targeted PET imaging from preclinical models to human application [34]. In multiple mouse tumor models, tracer uptake correlated strongly with measured GzmB levels and enabled early discrimination between responders and non-responders to immune checkpoint inhibitors, often preceding detectable changes in tumor size. Importantly, [^{68}Ga]Ga-grazytracer distinguished immunotherapy-induced pseudoprogression (high GzmB signal followed by tumor regression) from true progression (low GzmB signal). A first-in-human study in five patients with lung cancer or melanoma reported no acute adverse events. Notably, patients with positive post-treatment [^{68}Ga]Ga-grazytracer PET scans more frequently showed favorable clinical or metabolic responses, whereas negative scans were associated with poor outcomes, although the sample size was too small for definitive conclusions (Fig. 2). The authors also highlighted practical advantages, including same-day imaging with ^{68}Ga, straightforward kit-based synthesis, and potential applications in other GzmB-related settings (e.g., CAR-T therapy, transplant rejection, and immune-related adverse events). Key limitations included the very small clinical cohort and the need to optimize imaging timing given the transient nature of GzmB secretion.

Liu et al. conducted a prospective single-center study evaluating the diagnostic and predictive value of PET/CT with [^{68}Ga]Ga-NOTA-GSI, a GzmB–targeting radiopharmaceutical, for early response assessment to combination immunotherapy in advanced gastric cancer [35]. A total of 72 patients with stage III–IV disease receiving anti–PD-1 therapy plus chemotherapy were enrolled, of whom 40 were included in the final analysis. PET imaging was performed after the second or third treatment cycle. Quantitative parameters, including SUVmax, target-to-liver ratio (TLR), and target-to-blood ratio (TBR), were measured in primary tumors and metastatic lymph nodes, and response was evaluated according to RECIST (Response Evaluation Criteria in Solid Tumors) 1.1 criteria. Nineteen patients (47.5%) were classified as responders and 21 (52.5%) as non-responders. Responders exhibited significantly higher SUVmax, TLR,

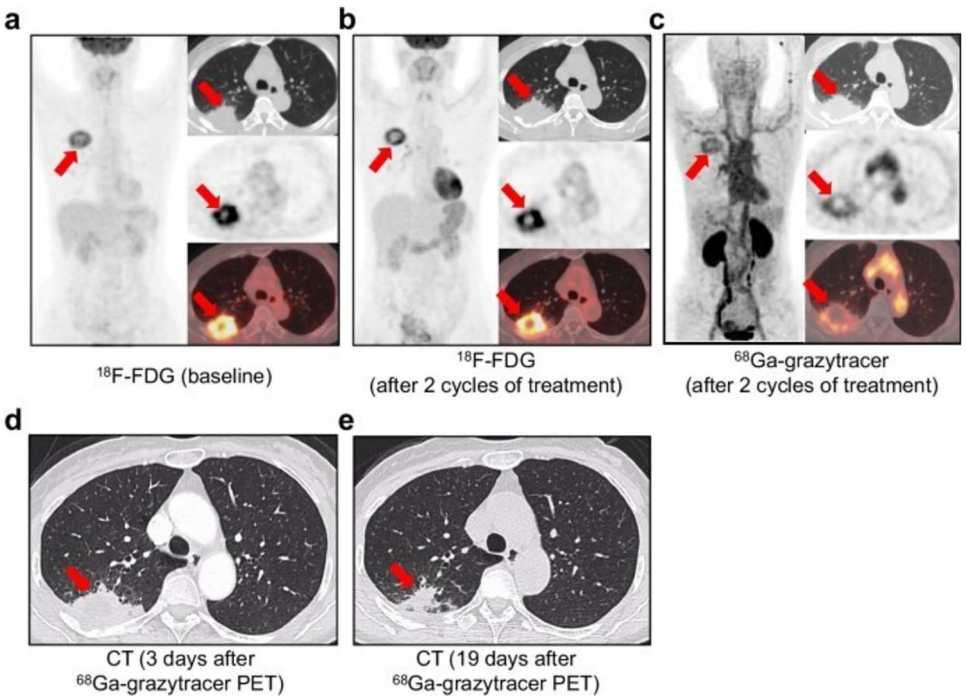

Fig. 2 **a** Baseline [^{18}F]FDG PET/CT demonstrated a mass in the right upper lung lobe, showing intense [^{18}F]FDG uptake (SUVmax 7.6). **b** After two cycles of combined immunotherapy and chemotherapy, [^{18}F]FDG PET/CT showed no change in tumor size; however, metabolic activity had increased compared with baseline (SUVmax 11.3 vs. 7.6). **c** [^{68}Ga]Ga-grazytracer PET/CT performed after two treatment cycles identified the right upper lobe mass with heterogeneously elevated tracer uptake (arrows; SUVmax 5.2). **d** Chest CT obtained 3 days following the [^{68}Ga]Ga-grazytracer PET/CT showed no change in lesion dimensions. **e** Chest CT performed 19 days after the [^{68}Ga]Ga-grazytracer PET/CT demonstrated a marked decrease in tumor size. Arrows denote tumor locations on the PET, PET/CT, and CT image. Reprinted from [34] under a creative commons attribution 4.0 international license (http://creativecommons.Org/licenses/by/4.0/). No changes were made

and TBR in both primary and nodal lesions. Receiver operating characteristic (ROC) analysis showed high diagnostic accuracy for both SUVmax and TBR calculated in tumors, with TBR-tumor emerging as an independent predictor of treatment response. No serious tracer-related adverse events were observed.

Shen et al. conducted a Phase 1/2 clinical trial evaluating the safety, biodistribution, GzmB specificity, and predictive value of [^{68}Ga]Ga-grazytracer PET/CT, including correlations with tumor immune phenotype [36]. Twenty-four immunotherapy-naïve patients (21 solid tumors, three lymphomas) received immune-checkpoint inhibitors ± chemotherapy or CAR-T (chimeric antigen receptor T-cell) therapy. Imaging included baseline and interim [^{18}F]FDG PET/CT and [^{68}Ga]Ga-grazytracer PET/CT after two to three cycles (or 1 month post-CAR-T). The tracer was safe, with no adverse events, with biodistribution imaging showing early renal uptake and rapid clearance. In seven surgical patients, tumor uptake correlated with GzmB staining. "Non-desert" (CD8-high) tumors had significantly higher SUVmax. Overall, 45.8% achieved complete or partial response, and these patients demonstrated significantly higher uptake. Of note, GzmB PET better predicted 6-month response and longer progression-free survival with respect to [^{18}F]FDG-PET and CT.

Table 2 summarizes the main results obtained in the preliminary studies of GzmB PET in cancer patients.

4 Translational Hurdles and Future Directions

GzmB PET uniquely targets a mechanistic end-point of cytotoxic immunity—the serine protease released by activated CD8$^+$ T cells and NK cells during target-cell killing—and therefore provides a proximal and functionally specific readout of immune effector activity rather than simply depicting immune-cell presence or nonspecific metabolic change. This biological positioning gives GzmB PET a conceptual advantage within the immune-imaging landscape, as it reflects the execution phase of antitumor immunity.

From a "strengths" perspective, GzmB PET offers several distinctive advantages. First, its biological specificity is notable: by imaging a terminal effector molecule of the granule-exocytosis pathway, it provides a direct link to tumor-cell killing that is conceptually closer to clinical benefit than surrogate measures such as glucose metabolism or bulk immune infiltration. Second, it enables an early functional readout of treatment efficacy. Multiple preclinical studies and first-in-human series report that tracer uptake increases in responding lesions before measurable tumor shrinkage or [^{18}F]FDG changes occur, thereby improving early response prediction and facilitating timelier therapeutic decisions. Third, rapid pharmacokinetics characterize most peptide-based GzmB tracers. Their fast renal clearance supports same-day imaging workflows and yields favorable tumor-to-background ratios compared with antibody-based immune-PET agents, which typically require delayed imaging because of prolonged blood-pool activity. Finally, GzmB tracers hold clear theranostic potential: identical peptide scaffolds can be labeled with diagnostic radionuclides (e.g., ^{68}Ga, ^{18}F) or therapeutic emitters (e.g., ^{90}Y, ^{177}Lu), enabling a seamless transition from target visualization to dosimetry-informed TRT. Notably, the integration of GzmB-targeted imaging with combined therapeutic strategies, particularly immune-checkpoint inhibitors and radioligand therapy, represents a promising avenue for precision oncology. Preclinical evidence suggests that TRT may enhance tumor immunogenicity through immunogenic cell death, thereby potentiating cytotoxic T-cell activation [37]. In this context, GzmB PET provides a unique opportunity to non-invasively monitor treatment-induced immune activation and to optimize sequencing and dosing of combination regimens. Furthermore, early changes in GzmB signal may serve as a pharmacodynamic biomarker to identify synergistic effects and guide adaptive treatment strategies

Despite these advantages, several limitations remain. A critical limitation of GzmB PET imaging relates to the dynamic nature of GzmB secretion and its extracellular diffusion. Because GzmB is released transiently and may rapidly diffuse or be internalized, PET imaging captures only a temporal snapshot of this process. Consequently, signal intensity is highly dependent on the timing of image acquisition relative to immune activation, underscoring the need for optimized and potentially longitudinal imaging protocols. Another challenge is the heterogeneity of signal sources. Although classically linked to cytotoxic lymphocytes, extracellular or perforin-independent GzmB activity and expression in certain non-cytotoxic populations, including subsets of myeloid or regulatory cells, may confound interpretation, particularly within inflamed or fibrotic tumor microenvironments. In this regard, it should be noted that most immunofluorescence and immunohistochemistry approaches detected total GzmB expression rather than its enzymatically active form. Despite this limitation, several studies have reported significant correlations between imaging-derived tracer uptake and ex vivo measurements of GzmB-positive immune cells.

It should be emphasized that most preclinical studies have been conducted in subcutaneous tumor models, which may not fully recapitulate the complexity of orthotopic or metastatic disease. The applicability of GzmB tracers to other anatomical sites depends on several factors, including tracer pharmacokinetics, tissue perfusion, and background uptake in organs such as liver and kidneys. These aspects may influence sensitivity and should be carefully considered

Table 2 Clinical studies of Granzyme B PET in oncology

Study (year)	Population and clinical setting	Tracer and imaging protocol	Validation/response assessment	Main quantitative findings	Translational interpretation and technical caveats
Zhou et al. (2022) First-in-human translational study; n = 5 [34]	Patients with lung cancer or melanoma on immunotherapy. No acute adverse events were reported after tracer administration.	[^{68}Ga]Ga-grazytracer. Same-day PET/CT was enabled by the 68Ga half-life; rapid renal clearance and kit-based synthesis were highlighted as logistical strengths.	Compared with baseline/follow-up imaging and clinical course; preclinical work showed correlation between tumor uptake and GzmB expression ($r \approx 0.72$). The tracer was used to explore pseudoprogression versus true progression.	Positive post-therapy scans were associated with subsequent favorable clinical/metabolic response, whereas negative scans tracked poor response. In preclinical validation, the tracer differentiated responders before tumor-size changes became evident.	Proof-of-concept clinical evidence for functional immune imaging. Main limitations: extremely small cohort, exploratory thresholds, and the need to optimize scan timing because GzmB release is transient.
Liu et al. (2024) Prospective, single-center study; June 2022-August 2023; 40/72 screened patients analyzed [35]	Stage III-IV advanced gastric cancer treated with anti-PD-1 therapy (sintilimab or camrelizumab) plus chemotherapy. Final cohort: 19 responders and 21 non-responders by RECIST 1.1.	[^{68}Ga]Ga-NOTA-GSI. Interim PET/CT was performed after the 2nd-3rd treatment cycle; quantitative analysis included primary tumor and metastatic lymph-node uptake.	Response defined by RECIST 1.1. Metrics: SUVmax, target-to-liver ratio (TLR), and target-to-blood ratio (TBR) for primary lesions and nodal metastases.	Responders showed significantly higher SUVmax, TLR, and TBR in both primary tumors and metastatic lymph nodes. ROC analysis: SUVmax-tumor AUC 0.886; TBR-tumor AUC 0.866; SUVmax cutoff 2.05 yielded 81.0% sensitivity, 84.2% specificity, and 82.5% accuracy. TBR-tumor was an independent predictor of response.	Most technically mature of the three clinical datasets because of its prospective design and prespecified quantitative readouts. Caveats: single-center experience, moderate sample size, and short-term/interim assessment rather than mature survival endpoints.
Shen et al. (2024) Phase 1/2 clinical trial; n = 24 immunotherapy-naive patients [36]	Mixed population: 21 solid tumors and 3 lymphomas; therapies included checkpoint inhibitors +/- chemotherapy and CAR-T in selected patients.	[^{68}Ga]Ga-grazytracer. Baseline and interim [18F] FDG PET/CT were paired with grazytracer PET/CT after 2-3 cycles of treatment or 1 month after CAR-T; early renal uptake and rapid clearance were described.	Response assessed with RECIST, Lugano, and PERCIST, depending on tumor type. Correlative pathology included GzmB immunohistochemistry in seven surgical patients; tumor immune phenotype was also evaluated.	Tracer uptake correlated with GzmB staining ($r = 0.77$ in surgical cases). Overall, 45.8% achieved CR/PR; responders had significantly higher uptake ($p = 0.0004$). ROC AUC for SUVmax was 0.899; a cut-off of 2.3 predicted 6-month response and longer PFS ($p = 0.0009$).	Strongest evidence for broad clinical utility across tumor entities and treatment classes. Caveats: heterogeneous diseases/therapies, small sample size, and interpretation challenges in pseudoprogression and CAR-T settings, despite excellent safety and no adverse events.

AEs adverse events, *AUC* area under the ROC curve, *CAR-T* chimeric antigen receptor T-cell therapy, *CR/PR* complete response/partial response, *FDG* fluorodeoxyglucose, *GzmB* granzyme B, *ICI* immune checkpoint inhibitor, *Lugano* Lugano classification, *PFS* progression-free survival, *PET/CT* positron emission tomography/computed tomography, *RECIST* Response Evaluation Criteria in Solid Tumors, SUV_{max} maximum standardized uptake value, *TBR* target-to-blood ratio, *TLR* target-to-liver ratio

in translational and clinical studies. It is worth mentioning, in fact, that absolute tumor uptake values reported across studies are relatively low (often < 1–2 % ID/g), which may impact sensitivity, particularly in lesions located in high-background regions. Therefore, optimization of tracer design and imaging protocols remains critical.

Clinically, potential indications include early response assessment to immunotherapy, patient stratification, detection of pseudoprogression, and monitoring of combination therapies. However, validation in real-world settings remains limited: published cohorts are relatively small and heterogeneous, while standardized sensitivity and specificity thresholds, cross-platform reproducibility, and correlations with long-term outcomes are still evolving. Dosimetry considerations are essential for both diagnostic and theranostic applications of GzmB-targeted tracers [38]. In this regard, one of the main limitations is that low tumor uptake may reduce the effectiveness of GzmB–targeted peptides in organs with higher background signal, particularly those involved in peptide excretion, such as the bladder and kidneys, and to a lesser extent the liver and intestines. In addition, the ability of GzmB–targeted peptides to stratify therapeutic response has been shown to be influenced by the background immune phenotype [39].

Looking ahead, several research and clinical priorities should be addressed to accelerate translation. At present, no single GzmB tracer can be definitively considered the lead candidate. However, peptide-based tracers such as [^{68}Ga]Ga-NOTA-GZP and [^{18}F]AlF-mNOTA-GZP have demonstrated the most extensive preclinical validation, while [^{68}Ga]Ga-grazytracer has shown promising early clinical results. Each agent presents distinct advantages in terms of pharmacokinetics, logistics, and translational readiness, and head-to-head comparative studies will be necessary to define their relative clinical value.

The lack of standardized quantitative metrics further complicates cross-study comparison and represents an important area for future methodological harmonization. Quantitative analysis of GzmB PET imaging remains an evolving area, with different studies adopting semi-quantitative metrics such as SUV (max, mean) and TBR. Each of these approaches presents specific advantages and limitations in the context of GzmB biology. SUV and SUV_{max} are widely used and allow standardized comparisons across studies; however, they provide a static snapshot of tracer uptake and may not fully capture the dynamic and spatially heterogeneous nature of cytotoxic immune activity. Tumor-to-background ratios (e.g., TBR or tumor-to-blood ratio) may offer improved robustness by accounting for systemic tracer distribution and background signal, which is particularly relevant for tracers with renal clearance or variable off-target uptake. However, these metrics depend on the choice of reference tissue and may introduce variability across studies. Given the transient secretion and diffusion of GzmB, static metrics may incompletely reflect true enzymatic activity. Importantly, the optimal quantitative approach for GzmB PET may differ from conventional oncologic PET imaging, as the underlying signal reflects a dynamic enzymatic process rather than steady-state tracer accumulation. Therefore, future studies should explore dynamic imaging approaches, kinetic modeling, and composite biomarkers to better capture the temporal dimension of immune activation.

On this path, standardization is essential: harmonized imaging windows, acquisition parameters, and quantitative endpoints must be defined across centers. In addition, prospective multicenter validation trials, ideally stratified by tumor type and therapy class, are needed to benchmark predictive performance against other well-established imaging modalities. Finally, kinetic and dosimetry studies integrating dynamic PET acquisitions with paired diagnostic-therapeutic administrations could translate tracer uptake into absorbed-dose estimates and optimize TRT scheduling. Finally, biological cross-validation through correlative tissue assays—including GzmB immunohistochemistry and single-cell immune profiling—will be crucial to map signal origin and resolve non-canonical sources of tracer uptake.

Integration with other immune imaging and molecular biomarkers will likely define the ultimate clinical value of GzmB PET. Rather than serving as a stand-alone modality, it should be viewed as a complementary data stream within a multiparametric framework. Direct comparisons and combinatorial algorithms should therefore be prioritized. For instance, pairing GzmB PET with [^{18}F]FDG can help distinguish metabolic flare from true progression. Likewise, stromal and microenvironment probes—such as FAPI for cancer-associated fibroblasts or macrophage-targeted tracers (e.g., TSPO/CD68)—can contextualize cytotoxic activity within the broader tumor ecosystem [40, 41]. Beyond imaging, cross-modality signatures that incorporate circulating biomarkers, including ctDNA or cytokine panels, will likely outperform single metrics for patient selection, early response assessment, and monitoring of complex combination regimens such as immunotherapy plus chemotherapy, CAR-T therapies, or TRT [42, 43]. Pragmatically, next-generation GzmB PET agents should be systematically compared with established immune-PET radiopharmaceuticals and tumor-microenvironment tracers to define relative strengths, interpretive pitfalls, and complementary clinical roles.

An additional crucial element could be the availability of GzmB-targeted tracers suitable for gamma camera imaging and single photon emission tomography (SPECT). This aspect is particularly relevant from an accessibility standpoint. While PET offers outstanding sensitivity and quantitative accuracy, its diffusion is still limited by high costs, complex infrastructure, and the need for dedicated

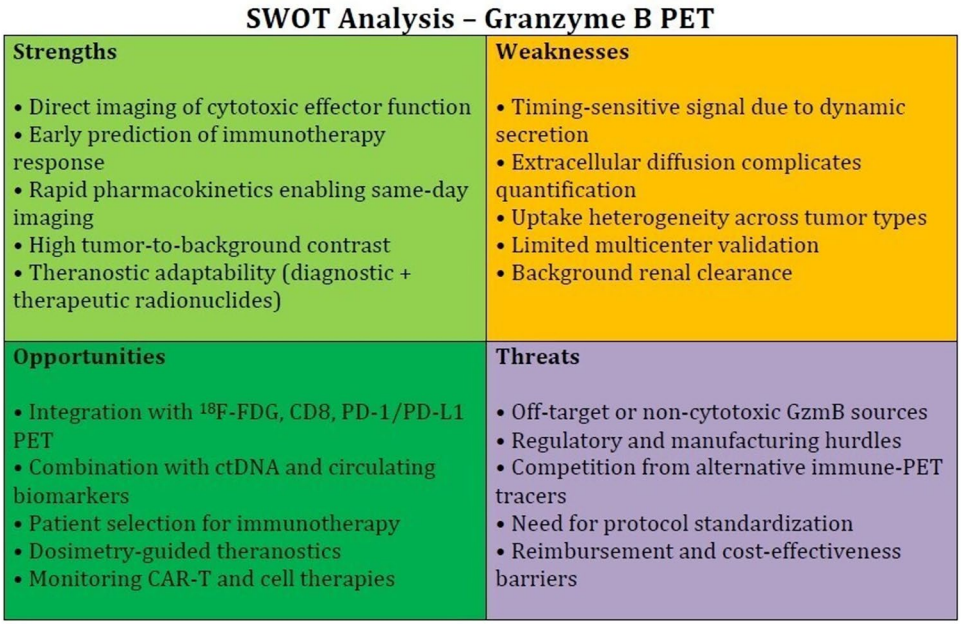

Fig. 3 SWOT framework (Strengths, Weaknesses, Opportunities and Threats) analyzing the potential of GzmB PET in clinical practice

radiochemistry facilities. Conversely, gamma cameras and SPECT systems are far more widely available across clinical centers [44–46]. The development of GzmB tracers compatible with these platforms could therefore broaden patient access to immune-functional imaging and facilitate implementation in routine practice. Although SPECT remains less sensitive than PET, ongoing technological advances continue to improve its diagnostic performance, making it a pragmatic and scalable complementary approach [47]. Figure 3 outlines the main advantages and disadvantages, as well as the potential translational opportunities, analyzed according to the SWOT framework (Strengths, Weaknesses, Opportunities and Threats).

5 Conclusions

GzmB PET provides a proximal, mechanism-based readout of cytotoxic effector activity and shows promising predictive performance in multiple preclinical models and early human studies. While peptide-based agents labeled with ^{68}Ga and ^{18}F have facilitated same-day imaging and translational evaluation, crucial challenges remain—including optimal imaging timing, signal heterogeneity from non-canonical sources of GzmB, and the need for multicenter validation and standardized quantitative thresholds. Integrated, prospective studies comparing GzmB PET with established immune-PET biomarkers and paired tissue/circulating correlative assays will be essential to define its role in clinical decision-making and potential theranostic applications.

Declarations

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