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REVIEW

New Vaccine Therapy for Triple-Negative Breast Cancer

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Abstract

Purpose of the review The objective of this review is to provide an analysis of early-phase clinical trials investigating vaccine therapies for triple-negative breast cancer (TNBC). Specifically, the focus is on ongoing trials that are actively recruiting or in progress, while excluding vaccines that target neoantigens or those that have already completed trials.

Recent findings Over the past decade, notable transformations have occurred in the strategy of breast cancer vaccine design. Traditional approaches to identifying tumor antigens, such as SEREX, have been replaced with modern techniques, such as RNA sequencing, HLA typing, and immunoinformatics. These new methods enable the identification and characterization of tumor antigens. Notably, current clinical investigations into tumor targets extend beyond mutated self-proteins or proteins that are overexpressed following neoplastic transformation. Clinical researchers are currently examining protein targets associated with cancer stem cells or non-malignant immune regulatory cell types within the tumor microenvironment. However, the application of up-to-date antigen delivery methods for certain types of breast cancer vaccine therapies still lags behind. Another significant transformation in comparison to previous breast cancer vaccine therapies is the emphasis on stimulating robust T-cell responses against breast cancer cells, independent of any B-cell response directed at the tumor.

Summary In conclusion, we critically assessed the tumor antigens targeted by vaccine immunotherapies in these new clinical trials, the delivery methods used for these antigens, and conclude by discussing potential future directions for the development of new TNBC vaccine therapies.

Keywords AE37 · Alpha-Lactalbumin · Clinical trial · TPIV200 · PVX-410 · TNBC · TriAdeno · STEMVAC · Survivin · BIRC5 · Adjuvanted microsphere vaccine · Peptide vaccine · Vaccine therapy

Introduction

Triple-negative breast cancer (TNBC) constitutes approximately 15%–20% of all breast cancer cases, is aggressive and has a high fatality rate approaching 40%. Aberrantly low expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2) in tumor tissue of patients with TNBC elevates the risk of recurrence and progression and represents a biomarker associated with a poor prognosis. TNBC cannot respond to conventional hormone receptor targeted therapies and thus its primary treatment lies in chemotherapy and surgical excision. Unfortunately, the use of conventional chemotherapeutic agents (e.g. paclitaxel, anthracycline, and alkylating agents) is associated with significant toxicity and comorbidities. Further complicating TNBC management is the heterogeneity of gene expression within tumor cells [1] and the lack of a unified diagnostic standard for TNBC molecular typing [2].

The limited treatment options for recurrent TNBC have traditionally been cytotoxic chemotherapeutic agents due to the absence of molecular targets expressed in more common types of breast malignancies. There is ongoing exploration of adjuvant immunotherapy for TNBC, with various categories of approaches being investigated [3]. One such approach, recently approved by the FDA for advanced TNBC, combines chemotherapy with immune checkpoint inhibitors (ICI) in order to enhance the existing adaptive

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immune response to breast tumor antigens. Clinical and histochemical data have demonstrated the effectiveness of anti-PD-1 mAb ICI as a neoadjuvant and adjuvant treatment for advanced TNBC patients who exhibit PD-L1 expression in their tumors and/or stromal cells (including tumor infiltrating lymphocytes). The subgroup of patients in the KEYNOTE-355 clinical trial who were PD-L1-positive demonstrated improved overall survival, whereas the PD-L1-negative subgroup fared poorly irrespective of anti-PD-1 treatment [5]. However, studies using immunohistochemistry have revealed that PD-L1 expression is only present in approximately 20% of TNBC cases [4]. This suggests that alternative approaches to adjuvant immunotherapy are needed. Notwithstanding the success of checkpoint inhibition (CPI) therapy in TNBC [6], vaccine-based immunotherapy of TNBC is an important emerging therapeutic option.

Neoplasms can be categorized into three immunophenotypes: "hot," "variable," and "cold," based, in part, on the distribution and presence of T cells and other immunocytes within the tumor microenvironment (TME) and specific characteristics of the TME itself [7]. The concept of "immune contexture" in human malignancies—a thorough analysis of the correlation between patient survival and the type, density, functionality, and placement of immune cells—has been comprehensively reviewed in the literature [8, 9]. Another determinant of a tumor's immunophenotype, or its Immunoscore, pertains to the molecular traits of the tumor cells themselves, particularly the degree of nuclear aberration indicating genomic instability, microsatellite instability, or defects in mismatch repair mechanisms. Therefore, "Hot tumors" are distinguished by a TME abundant in tumor-infiltrating lymphocytes (TILs), frequently alongside PD-L1 overexpression, genomic instability, and preexisting anti-tumor immune responses, contrasting sharply with "cold tumors," which lack immune cell infiltration.

Tumor-infiltrating lymphocytes (TIL) are found most extensively in triple-negative breast cancer (TNBC) compared with other breast tumor subtypes [10]. The penetration of CD8 + T cells into the tumor nests of TNBC, beyond their accumulation in the outer stroma, correlates positively with improved patient survival outcomes [11]. TNBC's classification as a "hot tumor" arises from its distinct characteristics, including the following:

- **Genomic Alterations and Molecular Diversity**: TNBCs are marked by a broad spectrum of genomic alterations and molecular heterogeneity, potentially enhancing their detectability by the immune system [12].
- **Homologous Recombination Repair Deficiency**: A notable fraction of TNBCs exhibit a deficiency in homologous recombination repair (HRR), which is often due to mutations in BRCA1 or BRCA2. This defect not only adds to the malignancy's virulence but also to its immunogenicity, as DNA damage accumulation can lead to neoantigen production [13].
- **High Proliferative Activity and Aggressiveness**: Characterized by rapid growth and genetic instability, most TNBCs may foster mutations that manifest as neoantigens recognizable by the immune surveillance system [12, 13]. The initial detection of MHC Class I presented neoantigens by "first responder" T cells that can trigger cytokine release, subsequently attracting more T cells and antigen presenting cells.
- **Elevated Immunological Infiltrate**: Compared with other types of breast cancer, TNBCs generally display a more substantial immunological infiltrate, including a higher prevalence of TILs. This feature is indicative of "hot" tumors and correlates with an enhanced response to specific therapies, like chemotherapy and immunotherapy [14].
- **Checkpoint Molecule Expression**: The frequent expression of checkpoint molecules, such as PD-L1, in TNBCs suggests a dynamic interaction with the immune system. Although these molecules can sometimes dampen the immune response, their expression also denotes an active immune milieu within the tumor, a trait leveraged in treatments utilizing checkpoint inhibitors to bolster the immune attack on the cancer [15].

The immunogenic profile of TNBC, distinguished by extensive immune cell infiltration, genomic heterogeneity, and neoantigen presence, not only sets TNBC apart from other breast cancer subtypes but also guides its therapeutic approaches, underscoring the pivotal role of immunotherapy and the pursuit of tailored treatments such as vaccine therapies aimed at its unique molecular and immune characteristics.

Vaccine technology has advanced significantly over the past two decades, introducing various methods for immunization, each with its own benefits and challenges. A simplified overview follows:

mRNA Vaccines: These vaccines deliver messenger RNA (mRNA) into cells, directing them to produce proteins or protein fragments that activate the immune system. They don't contain live viruses, reducing the risk of disease from the vaccine. However, they must be carefully designed, run the risk of miss-translation and must be stored at very low temperatures.

DNA Vaccines: These work similarly to mRNA vaccines but use DNA to carry the genetic information for antigens. The DNA is converted into mRNA inside the body's cells, initiating an immune response. They are more stable than mRNA vaccines and can be stored at room temperature, but there are still concerns about their safety and effectiveness in humans, particularly regarding

the potential integration of foreign DNA into the human genome.

Whole Protein Vaccines: This traditional approach uses recombinant pathogen or tumor antigens to trigger immunity. They are usually safe but their development and production are more complex and slow. The whole protein may contain pieces (epitopes) which do not provoke immunity and divert the attention of the immune system.

Viral Vector Vaccines: These utilize a harmless virus to deliver genetic material from the pathogen into human cells, which then produce a response from the immune system. They can create a lasting immune response but may not work as well in individuals who have immunity against the virus used as the vector.

Peptide Vaccines: These involve short peptides (parts of proteins) from the pathogen to stimulate an immune reaction. They are specific and quick to produce, but they might not provoke a strong enough immune response on their own and often require additional substances (adjuvants) to boost effectiveness.

The clinical trial data for the vaccine therapies that we present here all involve primary treatment for newly diagnosed patients and/or adjuvant treatment to avoid disease recurrence. NCT04674306 describes a three-arm clinical trial for a vaccine targeting Alpha-Lactalbumin addressing both of these approaches and also including a third arm for primary prevention in women at high risk for developing TNBC. Exclusion criteria for the primary prevention arm include lactating or pregnant women. Other vaccines discussed in this review which target self-proteins expressed in healthy adult tissue and are not tumor specific or overexpressed in tumors (e.g., TPIV200, STEMVAC, PVX-410) run the theoretical risk of provoking autoimmunity, suggesting that their use may need to be reserved to use as therapeutic vaccines rather than a prophylactic one. Perhaps with more safety data, the use of such vaccines could also be extended to at-risk individuals for development of developing breast cancer or having recurrence.

A number of vaccine therapies for advanced breast cancer have been tested in large Phase II or Phase III clinical trials, but none have yet shown significant therapeutic potential [16]. One early strategy in the development of breast cancer vaccines was to administer antigens that induce strong antibody responses directed against unique carbohydrate antigens expressed by tumor cells. For example, the Theratope vaccine, a synthetic mimic of the mucin-associated glycan epitope STn conjugated to keyhole limpet hemocyanin (KLH), a foreign antigen. In a 2008 double-blind randomized Phase III trial designed to evaluate time to progression and overall survival, the Theratope vaccine failed to prolong the mean time to progression in a study population of over 1000 metastatic breast cancer patients [17]. In 2016, another carbohydrate-directed vaccine targeting Globo H was tested in a large trial of patients with metastatic breast cancer [18]. In this study, the difference in progression-free survival between vaccinated and unvaccinated controls was not statistically significant, but the levels of Globo-H-specific antibodies in vaccinated patients were correlated with progression-free survival. Retrospective analysis of these and similar studies led clinical investigators to conclude that the trial failures might be associated with disease heterogeneity and advanced disease stage [19–21].

Over the last decade, the approach to designing breast cancer vaccines has undergone significant changes. Earlier methods such as SEREX [22], which were used to find and define tumor antigens, are being replaced by more modern techniques such as RNA sequencing, HLA typing, and the use of immunoinformatics tools [23]. Current vaccines are being developed to prompt a strong T-cell attack on breast cancer cells, independent of any B-cell response that targets the tumor. This article reviews the ongoing early-phase clinical trials (phases 1 and 2) for TNBC vaccines as of February 2024. Specifically, it examines trials that are actively recruiting or underway, as summarized in Table 1. It does not include vaccines that target neoantigens or those whose trials have already been completed, as these have been extensively reviewed elsewhere [24••]. This review critically examines the selected antigens for targeting by these vaccines (illustrated in Fig. 1) and their methods of delivery, and concludes with potential future directions for the development of new TNBC vaccines.

TNBC Antigen Targets and their Delivery Platforms

TPIV 200 Vaccine Targets Folate Receptor Alpha

Folate receptor alpha (FRα) is expressed in normal tissue involved in the uptake or concentration of folic acid (e.g. kidney proximal tubules and choroid plexus). FRα is highly overexpressed in certain epithelial cancers, such as TNBC [43] and ovarian cancer [44]. Of note is that many breast cancer patients show significant levels of spontaneous T-cell and antibody-immune responses to FRα antigens relative to healthy controls. Using T cells from these patients, five HLA class II-binding peptides from the FRα amino acid sequence eliciting T-cell immune responses were identified [45]. The combination of TPIV 200 and Durvalumab (anti-PD-1) has already been tested in ovarian cancer (NCT02764333).

For vaccination, the five peptides were dissolved in 1% DMSO, and rh GM-CSF was added to the mixture. In the NCT03012100 trial, patients will be vaccinated

Table 1 Current (2/2024) TNBC Breast Cancer Vaccine trials (active, recruiting, or not yet recruiting / phase 1 or 2)

Trial number (Trial Type)	Vaccine Name Antigen target [reference]	Whole protein/ peptide/other [reference]	Delivery route /Adjuvants [reference]	Used with Anti-PD-1, anti PD-L1, or anti CTLA-4	Company Information
NCT03012100 (adjuvant treatment)	TPIV200 Multi-epitope Folate Receptor Alpha Peptide Vaccine [25, 26]	4 bare HLA class II binding peptides from folate receptor alpha	Intradermal injection of bare peptide with granulocyte macrophage colony stimulating factor (GM-CSF) [27]	No	Marker Therapeutics Houston, TX, USA
NCT04674306 (primary treatment, adjuvant treatment, primary prevention)	Alpha-Lactalbumin Rh Lactalbumin) [28, 29]	Whole protein	Subcutaneous Injection of protein in Montanide ISA 51 and Zymosan (dose limiting AE) [28]	No	Anixa Biosciences San Jose, CA USA
NCT05504707 NCT04348747 (primary treatment)	HER peptide -DC HER2 primed DC HER 3 / ERB B3 primed DC [30]	Peptides MHC class II Multi alleles [31]	Lymph node injection of autologous dendritic cells pulsed with bare peptide [32]	With or without Pembrolizumab	In House: Lee Moffitt Cancer Center and Research Institute, Tampa, FL USA
NCT04024800 (primary treatment)	AE37 vaccine AE36 hybrid peptide (aa776-790), which is derived from the intracellular portion of the HER2 protein, and the core portion of the MHC Class II invariant chain (the Ii-Key peptide) [33, 34]	Hybrid Peptide binding Multiple MHC class II Alleles. [34]	Intradermal injection of bare peptide with GM-CSF	Yes Pembrolizumab	Multiple International University Collaborators Peptides made by GMP peptide contract manufacturers
NCT03387085 NCT02826434 (adjuvant therapy)	TriAdeno vaccine CEA (ETBX-011 – Ad5 vector) Brachyury (ETBX-051 -Ad5 vector) MUC1 (ETBX-061 Ad5 vector) Mutant RAS (GI-400**0** HI yeast) [35] CEA (GI-6207 HI yeast) [36] Brachyury (GI-6301 HI yeast) [37, 38]	Whole protein	Subcutaneous Injection Both Ad5 viral vector and heat inactivated yeast considered as adjuvants Hiltonol adjuvant used in the boost phase. Few AE [37]	Yes Avelumab Durvalumab	ImmunityBio Culver City, CA USA
NCT03362060 (primary treatment)	PVX-410 X-box binding protein 1 (XBP1), syndecan-1 (CD138), and CS1	4, 9-merPeptides HLA A2 restricted	Intramuscular injection with bare peptide and poly-ICLC (Hiltonol) in Montanide [39] vehicle	Yes Pembrolizumab	OncoPep, Inc Cambridge, MA USA
NCT05455658 NCT02157051 (adjuvant therapy)	STEMVAC epitopes of CD105 (Endoglin) [40], Y-box binding protein 1 (Yb-1) [41], SRY-box 2 (SOX2), cadherin 3 (CDH3), and murine double minute 2 (MDM2) proteins	Plasmid DNA	Intramuscular injection of plasmid DNA with GM-CSF [42]	No	In House: University of Washington Medical Center Cancer Vaccine Institute Seattle, WA USA

intradermally at 28-day intervals for six doses and then at 6-month intervals for an additional seven doses.

Recombinant Human α-Lactalbumin Vaccine

α-Lactalbumin is a "retired" self-protein that is exclusively expressed in breast tissue and only during late pregnancy and lactation. Over 70% of TNBC tissue samples express mRNA for α-Lactalbumin [29]. This figure is based on bulk sequencing of tumor mRNA, and the heterogeneity of expression of α-Lactabumin in TNBC, as might be determined by single cells RNA-seq, has not been reported [1, 46]. Expression of another secretory protein, mammaglobin-A, in TNBC tissue, as determined by immunohistochemistry, is heterogeneous [47].

In the NCT04674306 escalating dose safety trial, patients diagnosed with TNBC will be given three doses (with escalating concentration of peptide antigen) of vaccine at 3-week intervals. The purified recombinant α-Lactabumin whole protein was administered with a GMP-grade zymosan adjuvant in the Montanide ISA 51 VG vehicle.

HER Peptide- Loaded Autologous Dendritic Cell Vaccines

Perhaps counterintuitive to the definition of TNBC [48], the DecipHER trials (NCT05504707 and NCT04348747) aim to treat patients with brain metastases from TNBC with synthetic peptide antigens from the HER2 protein loaded onto autologous dendritic cells.

In a previous phase II trial of 275 HLA-A2-positive patients with HER2-low-expressing or triple-negative breast cancer treated with surgery, two study arms were created. In one arm, patients were treated with humanized monoclonal antibodies against HER2 (i.e. trastuzumab (Herceptin)) and GM-CSF alone. In the other arm, patients received the HER2-derived bare peptide (E75) vaccine nelipepimut-S (NPS), GM-CSF, and trastuzumab. The HER E75 peptide corresponds to an immunogenic HLA A2/A3 restricted cytotoxic T cell peptide epitope of HER2 (KIFGSLAFL, HER2/neu, p369-377) [49]. The outcome measure in this study was 24-month disease-free survival (DFS), and no significant difference was noted in 24-month DFS between the peptide vaccine and control treatment groups. Nevertheless, in the TNBC patient subgroup, DFS was significantly improved in patients receiving the HER peptide vaccine [50]. This and similar studies provide a rationale for adjuvant immunotherapy of TNBC with HER-based vaccines.

Technically simple leukapheresis/elutriation of cancer patients can be used to obtain peripheral blood monocytes. Maturation of PBMCs can be induced through cytokine exposure, driving their differentiation into populations of dendritic cells (DCs) which are suitable for clinical use [51]. DCs are known as powerful antigen presenting cells in the periphery and can serve as an ideal living peptide delivery vehicle to provoke T cell immunity against breast cancer [52]. This can be done through incubating DCs with tumor antigens either as tumor lysates, recombinant proteins, or HLA-Class I or II restricted synthetic peptide antigens. cDC1 are particularly effective APCs specialized in the production of interferon-γ and activation of CD8 + T cells through the cross-presentation of exogenous antigens [53].

The DecipHER trials deviated slightly from the use of the HLA-A2/A3 restricted E75 peptide as antigen, instead using other peptides selected from the primary amino acid sequence of HER2 and HER3 that have been predicted to bind to HLA class I and II molecules [31, 32] to be loaded onto autologous DCs. In these trials, the HER2/HER3-targeting DC vaccine is combined with anti-PD-1 mAbs (e.g. pembrolizumab) for the treatment of brain metastases from TNBC. During the induction phase, patients will receive anti-HER2/HER3 DC vaccine intradermally (ID) on days 1, 22, and 43 as well as intravenous infusion of pembrolizumab on the same day. During maintenance, patients will receive repeat pembrolizumab infusions every 21 days and optional booster doses of the anti-HER2/HER3 dendritic cell vaccine.

AE37 Synthetic Peptide Vaccine

The heart of the AE37 vaccine is a peptide hybrid of a HER2-derived peptide, AE36 (aa776-790), and a portion of the MHC Class II invariant chain (Ii), the LRMK Ii-Key core segment. This hybrid peptide is given with granulocyte–macrophage colony-stimulating factor (GM-CSF). Previous in vitro or preclinical studies identified an intracellular domain of HER2 (777–789) able of provoking a robust CD8 + cytotoxic T cell response [54, 55]. The immunogenicity of this peptide was attributed to its capacity to induce both HLA class I restricted responses and HLA class II restricted T cell help [56, 57].

The other portion of the AE37 hybrid peptide originates from the MHC Class II invariant chain (Ii), a protein that binds to newly minted MHC Class II molecules in the ER to prevent the binding of peptides destined for presentation by MHC Class I molecules. Only a small portion of Ii (aa 77–80 (LRMK) is needed to regulate the opening of MHC class II peptide binding groove. When the immunogenic HER peptide (AE36) is fused to the Ii (LRMK) peptide, the AE37 vaccine peptide is obtained [33].

For clinical use in the experimental treatment of TNBC patients (e.g. NCT04024800), AE37 will be administered intradermally as a bare peptide in three 21-day cycles for 5 cycles. Pembrolizumab will be given concomitantly with bare peptide, but then extended for an additional 30 cycles.

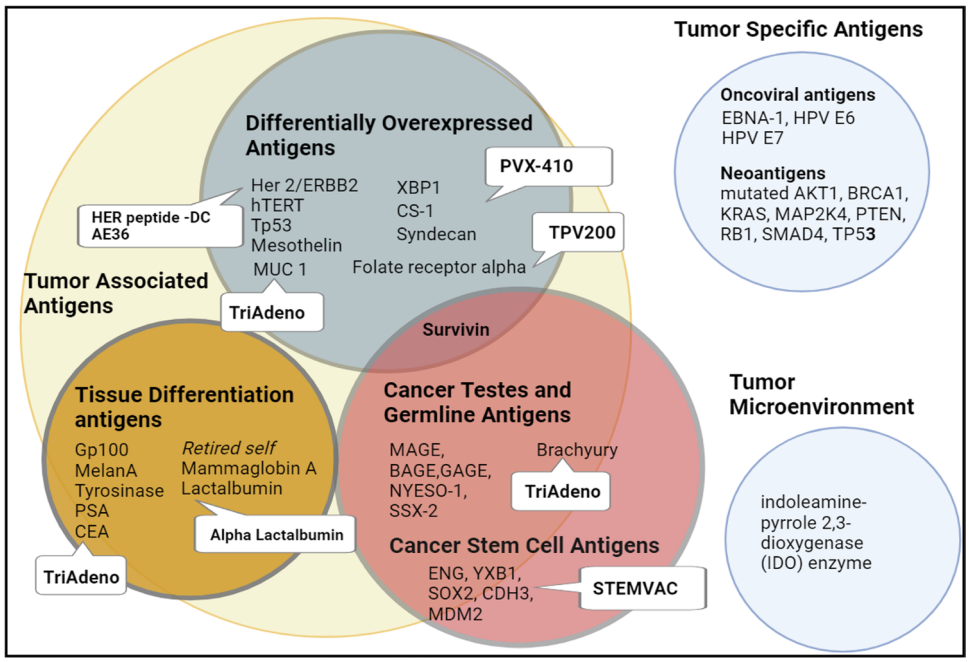

Fig. 1 TNBC vaccine antigen landscape in current clinical trials (2/2024). Tumor-associated antigens are categorized by their developmental expression patterns. The generic name or trade name is given in the white call-out boxes. The abbreviated gene names used are AKT1—AKT Serine/Threonine Kinase 1, BAGE—B Melanoma Antigen, Brachyury—T-Box Transcription Factor T, BRCA1—BRCA1 DNA Repair Associated, CDH3—Cadherin 3, CEA—Carcinoembryonic Antigen, CS-1—cell-surface glycoprotein CD2 subset-1 (CS1), also known as CD319, EBNA-1—Epstein–Barr nuclear antigen 1, ENG – Endoglin, Folate Receptor – Folate Receptor Alpha (FOLR1), GAGE—Cancer/Testis Antigen 4.1, Gp100—Premelanosome Protein (PMEL), HER 2—Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2), HPV-E6—Human papiloma virus transforming protein E6, HPV-E7—Human papiloma virus transforming protein E7 hTERT—Telomerase Reverse Transcriptase, KRAS—KRAS Proto-Oncogene, GTPase, Lacatalbumin – Lactalbumin Alpha (LALBA), MAGE—Melanoma-Associated Antigen 1, Mammoglobin A—secretoglobin family 2A member 2(SCGB2A2), MAP2K4—Mitogen-Activated Protein Kinase Kinase 4, MDM2—Mouse Double Minute 2 human proto-oncogen, MelanA – Melanoma Antigen Recognized By T-Cells 1, Mesothelin—Mesothelin (MSLN), MUC1—Mucin 1, Cell Surface Associated, NYESO-1—New York esophageal squamous cell carcinoma 1, PSA—Prostate-specific antigen, PTEN—Phosphatase And Tensin Homolog, RB1—RB Transcriptional Corepressor 1, SMAD4—Mothers Against Decapentaplegic Homolog 4, SOX2—SRY (Sex Determining Region Y)-Box 2, SSX-2—Synovial Sarcoma, X Breakpoint 2, Syndecan – Syndecan 1 (SDC1), TP53—Tumor Protein P53, Tyrosinase—Tumor Rejection Antigen AB (TYR), XBP-1—X-Box Binding Protein 1, YXB1—Y-Box Binding Protein 1

Single Antigen Targeting Vaccines Used in Combination

The TriAdeno vaccine brings into practice the well-accepted concept that vaccination against several tumor antigens is likely to be more effective than one [58]. The TriAdeno vaccine used in the NCT03387085 study targets four tumor antigens: CEA, Brachyury, six common RAS mutations, and MUC1 using an adenovirus vector for the initial priming doses, and then switches to a yeast delivery vector for the boosting-maintenance phases.

i. CEA

CEA also known as CEACAM5, is a highly glycosylated protein. CEA is present at low levels in the colon, stomach, tongue, cervix, and prostate epithelia, and changes in its expression levels and patterns are indicative of neoplastic transformation [59].

In the NCT03387085 study, CEA was first delivered via ETBX-011 (Ad5 [El-, E2b-]-CEA(6D)), an adenovirus vector in which the El, E2b, and E3 gene regions were removed and replaced with a gene encoding CEA with the CAP1-6D mutation [60, 61]. The CEA with CAP1 mutation, an amino acid substitution in the native protein at position 610 (asparagine to aspartic acid), was designed to increase the binding affinity of an HLA-A2 restricted T cell epitope peptide antigen within CEA. Whether this is an effective design strategy for CEA vaccines remains controversial [62, 63]. Adenoviral vaccines are delivered in a saline vehicle.

ii. Brachyury

Brachyury is a transcription factor that induces the endothelial to mesenchymal transition in human carcinoma cells [64]. Overexpression of Brachyury has been reported in TNBC [65], and in other cancers [66]. The Brachyury tumor antigen is delivered

via ETBX-051 (Ad5 [El-, E2b-]-Brachyury). ETBX-051 is the Ad5-based vector described above with the insertion of a modified human Brachyury gene. The sequence encoding the human brachyury protein was modified by introducing the enhanced-affinity HLA-A2 T-cell epitope (WLLPGTSTV) [67] and removing a 25-amino acid fragment involved in DNA binding.

iii. MUC1

MUC1 promotes the migration and invasion of various cancers. It is also involved in the regulation of pathways of cancer cell growth and apoptosis. However, it is overexpressed and poorly glycosylated in most human adenocarcinomas. In addition, aberrantly high levels of MUC1 have been reported in invasive lung cancers, pancreatic adenocarcinomas, prostate, and breast tumors, including TNBC [68]. The MUC1 tumor antigen is delivered again with an Ad5-based vector carrying the insertion of a modified human MUC1 gene (ETBX-o61). The modified MUC1 gene contains agonist epitopes(HLA-A2 restricted) designed to increase CTL antitumor immune responses [36].

In the second maintenance phase of the vaccination protocol, rather than using an adenovirus vector, a yeast vector is deployed. The use of recombinant Saccharomyces cerevisiae yeast as a vaccine delivery platform has been reviewed elsewhere [69••], but one important aspect of this delivery agent is its potential to break immune tolerance [70]. One of the TriAdeno vaccine components (GI-4000) contains four separate yeast populations, each carrying 2–3 different mutated RAS proteins with amino acid substitutions at different positions (i.e. [Q61R], [Q61L], [G12V], [G12C], [G12D] [Q61H] [Q61L], and [G12R]). Next, the potential anti-CEA response provoked with the Ad5-CEA administered in phase one is boosted with GI-6207, a heat-killed, recombinant Saccharomyces cerevisiae engineered to express full-length CEA with CAP1 mutation. The GI-6301 yeast vector component expresses the full-length Brachyury protein with an appended N-terminal MADEAP (Met-Ala-Asp-Glu-Ala-Pro) motif to promote antigen accumulation within the vector. The GI-4000 biological product is formulated in phosphate-buffered saline (PBS) for injection and contains separately vialed heat-killed yeast at a dose of approximately 2 10^8 cells. The mutant RAS yeast product will be selected to contain the Ras mutation in the tumor. At each dosing visit, CEA, Brachyury, and mutant RAS carrying heat- inactivated yeast vectors are administered.

The interim results for the trial have been published [71]. Nine patents received 3 treatment cycles of induction phase vaccinations. Eight treatment-related adverse events were reported in 4 patients. The early efficacy results were as follows: 7/9 patients showed a disease control rate 78% (CR + PR + SD), and overall response rate of 56% (PR + CR) and two patients (22% CR) showed a complete response.

PVX-410 Multiple Antigen Targeting Vaccine

The PVX-410 peptide vaccine was originally developed for multiple myeloma (MM)patients carrying the HLA A2 class I allele [39, 72]. PVX-410 targets four antigens with four synthetic peptides representing HLA class I restricted T cell epitopes. Based on the TNBC expression pattern of the parent proteins targeted by PVX-410, a clinical phase 1,2 vaccination trial has been initiated (Table 1).

i. XBP1

XBP1 is a critical transcriptional activator of the unfolded protein response (UPR), which increases tumor cell survival under prolonged endoplasmic reticulum (ER) stress and hypoxic conditions. A significantly higher level of XBP1 gene expression was found in primary cancer cells from breast cancer patients or colon cancer patients compared with cells from healthy donors [73, 74]

ii. Syndecan (CD138)

CD138, also known as syndecan-1, is a heparan sulfate proteoglycan that was first detected in human myeloma cells. Syndecan is expressed on epithelia, pre-B plasma cells, and other malignant cells, including carcinomas, lymphoid malignancies, Hodgkin lymphoma, and non-Hodgkin lymphoma. The syndecan-1 core protein has three domains: cytoplasmic, transmembrane, and extracellular. The cytoplasmic domain is linked to cytoskeletal proteins and regulates cell anchorage and morphology. The extracellular domain contains heparan sulfate chains that bind to numerous soluble and insoluble molecules.

iii. CS-1

CS1 is a lymphocyte signaling molecule that is highly expressed on MM cells but absent from acute leukemias, B-cell lymphoma, and Hodgkin lymphomas. The CS1 antigen is not expressed by normal tissues or stem cells but is expressed at low levels on natural killer (NK) cells and a subset of T lymphocytes compared with malignant plasma cells [75].

The four bare peptides representing HLA class I restricted epitopes from both spliced and unspliced XBP1, Syndecan, and CS-1 were delivered by subcutaneous injection. Each peptide dose was accompanied by intramuscular injections of an adjuvant composed of polyinosinic-polycytidylic acid-poly-L-lysine with cellulose carboxymethyl ether and l-lysine homopolymer carriers in a Montanide emulsion [39]. Six weekly doses are given initially, with booster doses given on weeks 10 and 28.

DNA Vaccine Targeting Multiple Antigens Associated with Cancer Stem Cells

There is a surprising lack of published studies regarding the preclinical development of the plasmid DNA-based STEMVAC vaccine. Only after retrieval of the patent application can substantive information regarding STEMVAC be found [76]. STEMVAC is a plasmid-based DNA vaccine encoding a multi-antigen fusion protein composed of in silico predicted MHC class II binding peptides from proteins associated with cancer stem cells [77] (Fig. 2).

In an earlier trial, NCT02157051, STEMVAC was studied in a Phase I non-randomized clinical trial aiming to study the side effects and the most effective dose of the CD105/Yb-1/SOX2/CDH3/MDM2-polyepitope plasmid DNA vaccine. In a patient population with advanced HER2-negative (25% TNBC) breast cancer previously treated and in remission, there were few adverse events. In the current trial, NCT05455658, patients with TNBC (stage Ib-III) will receive intradermal STEMVAC vaccine with recombinant granulocyte–macrophage colony-stimulating factor every month for 3 months, followed by booster vaccinations at 3 and 6 months.

The antigens targeted by STEMVAC are:

1. **Endoglin (CD105).** CD105 is a transmembrane glycoprotein known to mediate angiogenesis and can be found differentially expressed on breast cancer associated fibroblasts [78] As a cancer stem cell marker, the expression of endoglin is associated with the mesenchymal phenotype and drives the tumor epithelial to mesenchymal transition associated with metastasis [79, 80]
2. **YB1 (YBX1).** Yb1 may be associated with the promotion or maintenance of the cancer stem cell phenotype. When YBX1 is knocked down in mouse embryonic stem cells, the gene expression patterns change to a more differentiated phenotype, suggesting that Yb1 may preserve stem cell character and continuous proliferation [41].
3. **SOX2 (SRY-box 2).** SOX2 proteins have been identified as essential for maintaining the pluripotency of breast cancer stem cells [81].
4. **Cadherin 3 (CDH3).** P-Cadherin is a calcium-dependent homophilic cell–cell adhesion molecule whose expression is confined to the myoepithelium in normal breast tissue, and its overexpression in breast tumor tissue is associated with poor prognosis [82]. In certain animal models, P-cadherin expression is thought to be important in the maintenance of an undifferentiated state of normal mammary gland tissue [83].
5. **Murine double minute 2 (MDM2) plays** a complex role in tumor biology. MDM2 has been found to facilitate cancer growth via its pro-angiogenesis, metastasis, and metabolic reprogramming properties. On the other hand, MDM2 is a direct negative of the TP53 tumor suppressor, enabling cancer cells to evade apoptotic signaling [84].

Aside from the safety concerns surrounding the risk of plasmid DNA integration into the host genome and the substandard transfection efficiency of bare plasmid DNA [85], we note that the antigens targeted in STEMVAC are all proteins expressed by normal adult tissues (e.g. pancreatic islets). Thus, it is possible that immune tolerance to one or more of the peptide epitopes included in STEMVAC may exist. Although the details are sparse, this appears to be not the case as high-dose STEMVAC vaccinations appear to induce IFN-g-secreting T-cell responses in some patients [42], even in the absence of checkpoint inhibitor monoclonal antibodies. The long-term safety of inducing autoimmunity to self-proteins, even if associated with breast cancer stem cells, remains to be established.

New Targets and Delivery Platforms

Tumor Microenvironment (TME)

Potential antigenic targets, not necessarily expressed on tumors, but rather in tissues admixed and surrounding tumors, have been recently reviewed [86••]. Briefly, in the

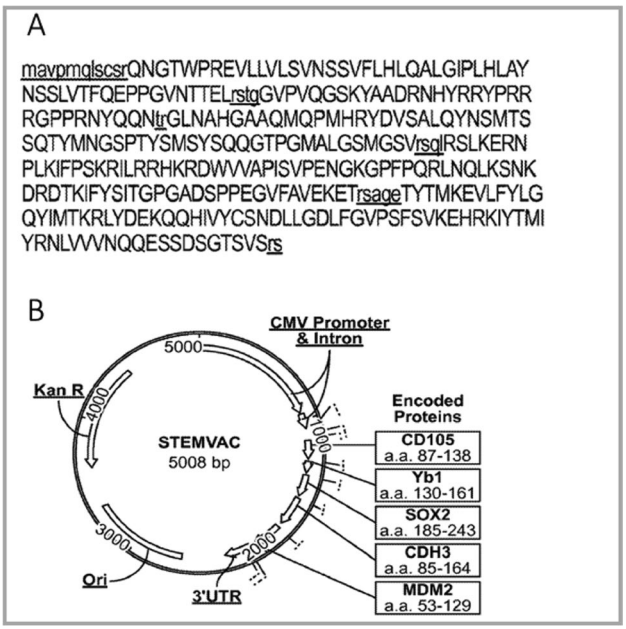

Fig. 2 Structure of STEMVAC DNA vaccine. Panel A. STEMVAC fusion protein vaccine antigen whose expression is driven intracellularly by the CMV promoter. The lowercase letters within the sequence represent cleavage sites that allow the fused multiple HLA class II binding peptides to associate with the MHC class II antigen binding groove. Panel B. Structure of the plasmid DNA carrying the STEMVAC fusion protein [76]

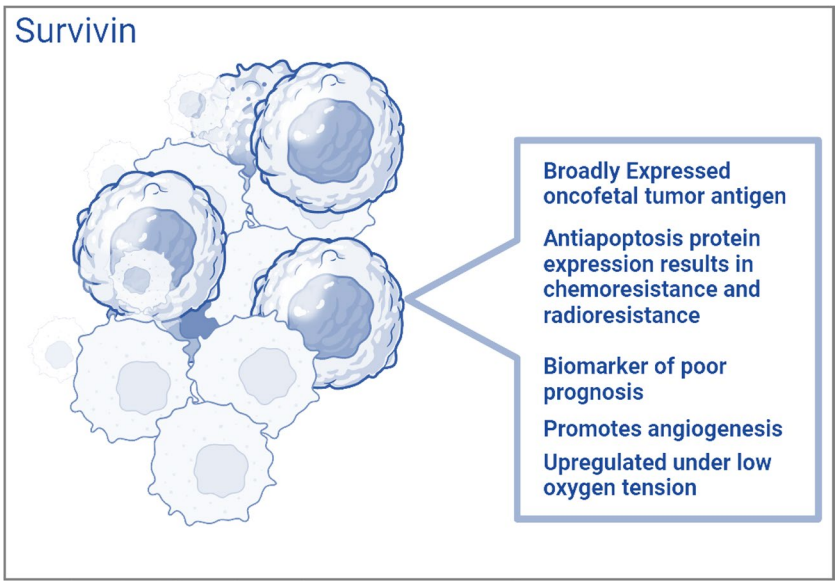

Fig. 3 Survivin (BIRC5) as a breast cancer tumor-associated antigen

TME, tumor metabolism often depletes essential nutrients and production of immune-suppressive metabolites. L-Tryptophan is an essential amino acid required for protein synthesis, and tryptophan metabolites directly suppress immune reactions. Enzymes such as indoleamine 2,3-dioxygenase (IDO) degradation tryptophan, and T cells respond to low tryptophan levels with arrested proliferation and proliferative arrest [87]. IDO is a self-antigen expressed in non-malignant tissues; thus, the finding that both CD4- and CD8-specific T cells can react to IDO-derived HLA-restricted epitopes is surprising [88, 89]. IDO-specific T cells lyse IDO + melanoma and leukemia cell lines.

Table 2 Unique characteristics of the microsphere vaccine platform relative to recombinant viral vaccines and bare peptide vaccines

Advantages	Limitations
Cost-Effectiveness: Readily synthesized and peptides purified at low cost, making them economically viable. Uses off-the-shelf reagents, simplifying the production process	HLA Type Restriction: Class I MHC restriction limits the relevance of individual peptides to certain HLA types, reducing universality
Stability: Stable at room temperature for more than 12 months, ensuring a longer shelf life	Immune Response: T cell immune responses may be transient and/or of low magnitude, potentially impacting long-term efficacy
Safety: Synthetic peptides have demonstrated safety in many human studies. The controlled release adjuvanted microsphere –short peptide vaccine used in this study has demonstrated safety in rodent and NHP models	Epitope Diversity: Peptide vaccine may have to include a large number of epitopes to confer a therapeutic effect across a wide range of patients
Specificity and Targeted Delivery: Using defined epitopes avoids uncharacterized antigens that may cause non-therapeutic or autoimmune activity. The microsphere diameter is optimized to target APCs with phagocytic properties and avoid nonprofessional nonphagocytic APCs. Avoids MHC binding competition from non-immunogenic epitopes	Induction of B cell antibody responses: At present, the microsphere platform is a "T-cell vaccine" only
Monitoring: Known MHC class I and class II epitope sequences enable direct monitoring of T cell responses, enhancing vaccine efficacy assessment	Inaccuracy of T cell epitope prediction methods: Bioinformatic T cell epitope prediction methods fall short of 100% accuracy in the absence of confirmational studies
Booster Vaccines: Feasibility of repeated booster vaccines to maintain or enhance immune responses No antivector immune response	
Protection: Peptide encapsulation in controlled release PLGA microspheres protects T cell epitopes from extracellular degradation	
Adjuvant Efficacy: Microsphere-encapsulated synthetic adjuvants promote optimal co-stimulation molecule expression by targeted APCs	
Mucosal Delivery: Adjuvanted microspheres are suitable for mucosal surface delivery by inhalation, broadening the application scope	

Table 3 Potential benefits of the multiantigen approach to cancer vaccination

Enhanced Immune Response: By presenting multiple tumor antigens, the immune response is broadened, potentially leading to a more effective attack on the tumor and reducing the chance of missing residual tumor cells. By including multiple antigens in a vaccine, the probability that a patient's immune system will effectively respond to at least one of the antigens is increased, potentially benefiting a broader range of patients.
Reduced Risk of Immune Evasion: Tumor cells frequently mutate, resulting in changes in their antigenic profile and evasion from immune detection and attack.
Targeting Heterogeneity: Tumor cells are often heterogeneous in their phenotypic profile, particularly in TNBC. A multi-antigen vaccine increases the chance of targeting this diversity within the tumor cell population, leading to a more effective treatment with less escape from immunosurveillance.

There are 2 active clinical trials with IDO MHC class I restricted peptide vaccines although none of them focus on TNBC (NCT03047928 and NCT05843448). Two of these trials administered the bare IDO peptide antigen in a Montanide adjuvant emulsion. In the NCT02785250 trial, rather than administering the bare peptide.

Adjuvanted Survivin Microspheres

The protein survivin (SVN, a.k.a BIRC5 shown in Fig. 3), is an inhibitor of apoptosis and overexpressed in many malignancies, such as breast cancer stem cells and breast tumor tissue [90], compared to adjacent normal adult cells and tissues [91, 92]. This indicates that SVN may be an ideal target in tumor cells, as recognized by the National Cancer Institute over 20 years ago [93]. Over the years, the role of SVN as an antigen in humoral and cellular immunity has been investigated in animal models and cancer patients. Several immunogenic B- and T-cell epitopes of SVN have been identified and utilized in the development of synthetic survivin-peptide cancer vaccines [94–97]. These studies now support ongoing clinical trials that aim to target SVN in diseases like glioblastoma, neuroendocrine tumors, ovarian cancer, and hormone receptor-positive breast cancer (e.g., NCT05163080, NCT02334865, NCT04895761). In glioblastoma, immunotherapy directed against survivin has shown to significantly extend overall survival compared to standard radiation therapy and chemotherapy [98].

Recently, adjuvant vaccine-based immunotherapy for triple-negative breast cancer (TNBC) has been reviewed [24••]. Out of the 42 clinical studies reviewed, only one exploratory human study has focused on targeting SVN in TNBC [99]. To explore novel SVN based immunotherapies for TNBC, we utilized the 4T1 murine model of TNBC [100] to assess the therapeutic potential of a synthetic SVN peptide-based microparticle vaccine for adjuvant immunotherapy. Our previous work involved the development of an adjuvanted, synthetic peptide-poly (lactic-co-glycolic acid) (PLGA) copolymer microparticle vaccine platform [101] capable of inducing strong therapeutic CD8 + cytotoxic T lymphocyte responses to viral antigens presented by MHC Class I molecules in non-human primate [102] and murine models [103]. MHC-restricted synthetic peptide epitopes were selected for this platform over whole proteins because synthetic peptide epitopes can outperform whole protein vaccines under certain condition [104]. Other advantages (and limitations) of this microsphere peptide antigen delivery platform are summarized in Table 2. In a preclinical murine model, we found that vaccination with adjuvanted survivin-peptide-loaded microspheres significantly inhibited the growth of orthotopically implanted tumors in a 4T1 murine model of TBNC [105]. We also showed in a clinical study that survivin is not expressed in normal breast tissue near survivin-expressing breast tumor tissue in women luminal breast cancer undergoing lumpectomy [106]. First-in-human clinical studies for this survivin-targeting microsphere vaccine are currently in the planning stage.

Conclusions

The breast vaccines listed in Table 1 introduce significant innovations in adjuvant immunotherapy compared with past cancer vaccines. A key advancement is their formulation to target multiple tumor antigens, offering benefits over the traditional approach of targeting a single antigen, as detailed in Table 3. Among the novel approaches in breast cancer vaccines is the targeting of retired self-antigens, like α-Lactalbumin and Mammaglobin A. These antigens can be considered a novel specific category within tumor-associated differentiation antigens, similar to MelanA and tyrosinase.

Another innovative strategy is focusing on antigens present in the tumor microenvironment. These antigens are not necessarily linked directly to tumors or specific to them, but they play a role in suppressing T cell immune responses. Targeting these antigens aims to transform "cold" tumor areas, which are less active immunologically, into "hot" ones that are more active, a change that has been correlated with improved survival rates for patients. However, there is a risk involved in targeting non-tumor-associated self-antigens (for example, using STEMVAC), as it could trigger unwanted autoimmune reactions, a concern also noted with checkpoint inhibition immunotherapy [107].

In the clinical trials listed in Table 1 that vaccinate patients with bare MHC restricted synthetic peptide tumor antigens, there may be ample room for improvement in the context of delivery platforms. In the past, the clinical efficacy of bare peptide vaccines has been poor, attributable to poor antigen presentation, limited serum half-lives of the synthetic peptides, and low immunogenicity [108]. Next-generation synthetic peptide antigen delivery platforms, such as liposomes, hydrogels, microemulsions, immune-stimulating complexes, and other nanoparticle or microparticle systems, have been explored in various vaccination applications [109••] and should be considered in the development of next-generation synthetic peptide vaccines.

Author Contributions P.H. and R.R. wrote the main manuscript text and P.H. prepared figures 1-3. All authors reviewed the manuscript.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests R.R. and P.H. are employees of Flow Pharma, Inc. a biotechnology company engaged in the development of a breast cancer vaccine therapy. R.R. and P.H. receive compensation in cash and stock from Flow Pharma, Inc. R.R. is a co-inventor of issued and pending patents directly or indirectly related to the topic of the submitted manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Research Involving Human Participants and/or Animals Not applicable.

Informed Consent Not applicable.

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- • Of importance
- •• Of major importance

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