



HER2-Positive Breast Cancer: Characterization of the Tumor Microenvironment and Development of an Innovative CAR-T Cell Immunotherapy Strategy

University of Barcelona
School of Medicine and Health Sciences
Doctoral Program in Medicine and Translational Research

Laura Angelats Company
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HER2-Positive Breast Cancer: Characterization of the Tumor Microenvironment and Development of an Innovative CAR-T Cell Immunotherapy Strategy

Doctoral thesis dissertation presented by
Laura Angelats Company

to apply for the degree of doctor at the University of Barcelona

Doctoral Program in Medicine and Translational Research
School of Medicine and Health Sciences

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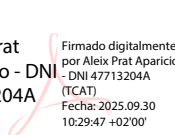
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A la meva família i a tu que m'has regalat la vida.

"Life is not easy for any of us. But what of that? We must have perseverance and,
above all, confidence in ourselves." –

Marie Curie

"The only way to do great work is to love what you do." –

Steve Jobs

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Abbreviations

A

ACT: adoptive cell therapy
ADC: Antibody–Drug Conjugates
AEs: adverse events
AOI: area of interest

B

BC: breast cancer
BCRs: B-cell receptors

C

CARs: chimeric antigen receptors
CBR: Clinical Benefit Rate
CDR: complementarity-determining region
CI: confidence interval
CIGGs: core IGG genes
CNS: central nervous system
CRS: cytokine release syndrome
CTCAE: Common Terminology for Classification of Adverse Events
ctDNA: circulating tumor DNA

D

D: Diversity gene
DCR: Disease-control rate
DLT: Dose-limiting toxicity
DOR: Duration of response
DSP: Digital Spatial Profiling

E

EBC: Early-stage breast
ECOG: Eastern Cooperative Oncology Group
EMA: European Medicines Agency
ESMO: European Society for Medical Oncology

F

FDA: Food and Drug Administration
FFPE: formalin-fixed paraffin-embedded

H

HA: high affinity
HCC1954: breast ductal carcinoma cell line
HER2: Human epidermal growth factor receptor 2
HER2+: HER2-positive
HR: Hazard ratio
H&E: hematoxylin–eosin

I

i: inhibitors
ICANS: immune effector cell-associated neurotoxicity syndrome
IGG: 14-gene immunoglobulin
IgH: immunoglobulin heavy chain
IHC: immunohistochemistry
IL-2: interleukin-2
IL-13R: interleukin 13 receptor
ITAMs: immunoreceptor tyrosine-based activation motifs

J

J: Joining gene

L

LCK: lymphocyte-specific protein tyrosine kinase
LA: low affinity
LV: lentivirus

M

mBC: metastatic breast cancer

M11: mesothelin

MTD: Maximum tolerated dose

N

ND: normal donor

NK: natural killer

O

ORR: objective response rate

OS: overall survival

P

PBMCs: peripheral blood mononuclear cells

pCR: pathological complete response

PFS: progression-free survival

PI3K: phosphatidylinositol 3-kinase

P/S: penicillin-streptomycin

R

RNA-seq: RNA sequencing

ROR: risk of recurrence

S

scFv: single-chain variable fragment

scRNA-seq: single-cell RNA sequencing

SHM: somatic hypermutation

SKOV3: ovarian cystadenocarcinoma

cell line

T

TCRs: T-cell receptors

T-DM1: trastuzumab emtansine

T-DXd: trastuzumab deruxtecan

TGF: transforming growth factor

TILs: Tumor-infiltrating lymphocytes

TIL-B: tumor-infiltrating B lymphocytes

TIL-T: tumor-infiltrating T lymphocytes

TKIs: Tyrosine kinase inhibitors

TLS: tertiary lymphoid structures

TME: tumor microenvironment

TNBC: Triple-negative breast cancer

TRAF: TNF receptor-associated factor

TRUCKs: T cells redirected for antigen-unrestricted cytokine-initiated killing

TU: tumor

U

UMAP: uniform manifold

approximation and projection

UTD: untransduced

V

V: Variable gene

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Doctoral thesis presented in the classic format, accompanied by an annexed article

This thesis seeks to provide a comprehensive characterization of the tumor microenvironment in HER2-positive breast cancer and to investigate novel therapeutic strategies.

STUDY 1 – IGG Project

Characterization of the tumor microenvironment in HER2-positive breast cancer.

The data presented in this report corresponds to the HER2-positive breast cancer samples mentioned in the following article:

Original research article: Angelats L, Paré L, Rubio-Perez C, et al. Linking tumor immune infiltration to enhanced longevity in recurrence-free breast cancer. *ESMO Open*. 2025; 10(1):104109. doi:10.1016/j.esmoop.2024.104109. IF: 8.3; Q1.

STUDY 2 – ARI-HER2

Optimizing CAR-T cell manufacturing to facilitate clinical translation in advanced HER2-positive breast cancer.

SUMMARY

HER2-positive breast cancer: characterization of the tumor microenvironment and development of an innovative CAR-T cell immunotherapy strategy

HER2-positive breast cancer (BC) represents approximately 15–20% of all BCs and is defined by the overexpression or amplification of the HER2 receptor. Although the development of HER2-targeted therapies has improved patient outcomes, particularly in the metastatic setting, more than 20% of patients eventually develop therapeutic resistance. This limitation highlights the urgent need to identify new biomarkers and therapeutic strategies to further improve survival. Within this context, we developed two research areas: the study of the 14-gene immunoglobulin (IGG) signature included in the HER2DX genomic test, and the development of HER2-directed CAR-T cell therapies.

The first project focused on understanding the biological role of the IGG signature. Previous findings associated this signature with improved responses to neoadjuvant anti-HER2 therapies and superior survival outcomes, although its precise biological meaning remained unclear. Based on this, we hypothesized that tumors with elevated IGG expression might be enriched in immune cell infiltrates with clonal diversity, which could modulate therapeutic response in early-stage BC. The main objective was to evaluate the role of the IGG signature and to elucidate the contribution of immune infiltrates in HER2-positive tumors. The results demonstrated that tumors with high IGG expression exhibited an immune-inflamed microenvironment characterized by plasma cells, T cells, and antigen-presenting cells, while also presenting reduced immune suppression and fibrosis. These tumors showed increased polyclonality of both B cells and T cells. Moreover, the IGG signature emerged as a scalable and minimally invasive method to infer the presence of tertiary lymphoid structures (i.e., the so-called TLS) directly from transcriptomic data, providing a valuable diagnostic advantage. Importantly, the combination of multiple IGG-related metrics further enhanced prognostic accuracy. Altogether, these findings establish the IGG signature as

powerful precision oncology tool, paving the way for therapeutic strategies that better address recurrence risk and longevity.

The second project addressed the limitations of conventional HER2 therapies by developing HER2-specific CAR-T cells. Unlike trastuzumab, trastuzumab emtansine (T-DM1), or trastuzumab deruxtecan (T-DXd), CAR-T cells kill tumor cells through direct induction of apoptosis. Given their distinct mode of action, CAR-T cells could provide an effective alternative for patients who develop resistance due to impaired antibody internalization, defective lysosomal degradation, or chemotherapy insensitivity. Building on prior work of our lab, a candidate CAR construct incorporating a 4-1BB co-stimulatory domain had already shown strong persistence and antitumor efficacy *in vitro* and *in vivo*. However, its expansion was limited by basal tonic signaling, a phenomenon that could compromise large-scale clinical production. We hypothesized that reducing this signaling could enhance CAR-T cell proliferation without impairing function. The main objective was therefore to optimize the production of HER2-BBz chimeric antigen receptor (CAR)-T cells and evaluate the toxicity of this construct for potential clinical application.

Experimental results demonstrated that the addition of dasatinib at 50 nM effectively reduced tonic signaling in 4-1BB CAR-T cells, leading to improved proliferation and reduced apoptosis during the expansion phase. Importantly, short-term exposure between days three and four of culture was sufficient to achieve optimal effects, as prolonged exposure offered no additional benefit in terms of expansion, CAR expression, functional activity, or transcriptomic profile. In functional assays, the HER2-BBz CAR could distinguish between high and physiological HER2 expression, maintaining strong antitumor activity while minimizing off-tumor toxicity. This selectivity expanded its therapeutic window relative to high-affinity HER2 CARs, supporting its potential clinical safety and efficacy. Collectively, these advances will enable the initiation of the ARI-HER2 clinical trial, a milestone in the application of CAR-T cell therapy to solid tumors.

In conclusion, both projects highlight complementary strategies for improving outcomes in HER2-positive BC. On one hand, the IGG signature provides biological insight into the role of immune infiltration and clonal diversity, emerging as a robust biomarker that can guide prognosis and therapeutic decisions. On the other, HER2-

specific CAR-T cells optimized through modulation of tonic signaling represent an innovative therapeutic alternative capable of overcoming resistance to conventional HER2-targeted treatments. Together, these findings underscore the importance of combining biomarker-driven approaches with novel immunotherapies to develop more personalized strategies for patients. The planned initiation of the ARI-HER2 clinical trial in 2025-2026 further reflects the potential of translating preclinical advances into benefits for individuals with HER2-positive BC.

Key words: CAR-T, HER2-positive breast cancer, tonic signaling.

RESUM

Càncer de mama HER2-positiu: caracterització del microambient tumoral i desenvolupament d'una estratègia innovadora d'immunoteràpia amb cèl·lules CAR-T

El càncer de mama HER2 positiu representa aproximadament un 15–20% de tots els casos de càncer de mama i es defineix per la sobreexpressió o l'amplificació del receptor HER2. Tot i que el desenvolupament de teràpies dirigides contra HER2 ha millorat els resultats en pacients, especialment en l'àmbit metastàtic, més d'un 20% dels pacients acaben desenvolupant resistència als tractaments. Aquesta limitació posa de manifest la necessitat urgent d'identificar nous biomarcadors i estratègies terapèutiques que permetin millorar encara més la supervivència. En aquest context, vam desenvolupar dues línies de recerca: l'estudi de la signatura d'immunoglobulina (IGG) de 14 gens inclosa en el test genòmic HER2DX, i el desenvolupament de teràpies amb cèl·lules CAR-T dirigides contra HER2.

El primer projecte es va centrar a comprendre el paper biològic de la signatura IGG. Estudis previs havien associat aquesta signatura amb millors respostes a les teràpies neoadjuvants anti-HER2 i amb una supervivència superior, tot i que el seu significat biològic continuava sent incert. A partir d'aquí, vam plantejar la hipòtesi que els tumors amb una elevada expressió d'IGG podrien estar enriquits en infiltrats de cèl·lules immunitàries amb diversitat clonal, fet que podria modular la resposta terapèutica en el càncer de mama en fases inicials. L'objectiu principal era avaluar el paper de la signatura IGG i estudiar la contribució dels infiltrats immunitaris en tumors HER2 positius. Els resultats van demostrar que els tumors amb alta expressió d'IGG presentaven un microambient tumoral inflammat, caracteritzat per la presència de cèl·lules plasmàtiques, limfòcits T i cèl·lules presentadores d'antigen, alhora que mostraven una menor immunosupressió i fibrosi. Aquests tumors també evidenciaven una major policlonalitat tant de les cèl·lules B com de les cèl·lules T. es va establir com una eina escalable i mínimament invasiva per a la inferència de la presència d'estructures limfoides terciàries a partir de dades transcriptòmiques, aportant un valor diagnòstic rellevant. Cal destacar que la combinació de múltiples mètriques

relacionades amb l'IGG va millorar encara més la precisió pronòstica. En conjunt, aquestes troballes consoliden la signatura IGG com una eina potent en oncologia de precisió, obrint el camí a estratègies terapèutiques més efectives per reduir el risc de recurrència i millorar la longevitat.

El segon projecte va abordar les limitacions de les teràpies convencionals contra HER2 mitjançant el desenvolupament de cèl·lules CAR-T específiques per HER2. A diferència del trastuzumab, T-DM1 o T-DXd, les cèl·lules CAR-T destrueixen les cèl·lules tumorals mitjançant la inducció directa d'apoptosi. Gràcies al seu mecanisme d'acció diferent, les CAR-T podrien oferir una alternativa eficaç per a pacients que desenvolupen resistència a causa d'una internalització defectuosa dels anticossos, una degradació lisosomal ineficaç o insensibilitat a la quimioteràpia. Un treball previ del nostre grup va demostrar que un constructe CAR amb un domini coestimulador 4-1BB tenia una forta persistència i eficàcia antitumoral tant *in vitro* com *in vivo*, però la seva expansió estava limitada per la presència de senyalització tònica, un fenomen que compromet la producció clínica a gran escala. Vam plantejar la hipòtesi que reduir aquesta senyalització podria millorar la proliferació de les CAR-T sense perjudicar-ne la funció. L'objectiu central va ser, per tant, optimitzar la producció de cèl·lules HER2-BBz CAR-T i avaluar la seva toxicitat per una possible aplicació clínica.

Els resultats experimentals van demostrar que l'addició de dasatinib a 50 nM reduïa eficaçment la senyalització tònica en cèl·lules CAR-T amb 4-1BB, millorant-ne la proliferació i disminuint l'apoptosi durant la fase d'expansió. És important destacar que una exposició de curta durada entre els dies tres i quatre de cultiu era suficient per aconseguir els efectes òptims, ja que una exposició més prolongada no oferia cap benefici addicional pel que fa a expansió, expressió del CAR, activitat funcional o perfil transcriptòmic. En assajos funcionals, el CAR HER2-BBz podia discriminar entre nivells alts i fisiològics d'expressió d'HER2, mantenint una potent activitat antitumoral i minimitzant alhora la toxicitat fora del tumor. Aquesta selectivitat va ampliar la seva finestra terapèutica en relació amb els CAR d'alta afinitat per HER2, donant suport al seu potencial de seguretat i eficàcia clínica. En conjunt, aquests avenços permetran la posada en marxa de l'assaig clínic ARI-HER2, un fet que representa una fita en l'aplicació de la teràpia CAR-T en tumors sòlids.

En conclusió, ambdós projectes destaquen estratègies complementàries per millorar els resultats en el càncer de mama HER2 positiu. D'una banda, la signatura IGG aporta una visió biològica sobre el paper de la infiltració immunitària i la diversitat clonal, consolidant-se com un biomarcador robust capaç de guiar el pronòstic i les decisions terapèutiques. De l'altra, les cèl·lules CAR-T específiques per HER2, optimitzades mitjançant la modulació de la senyalització tònica, representen una alternativa terapèutica innovadora capaç de superar la resistència a les teràpies convencionals dirigides a HER2. En conjunt, aquests resultats subratllen la importància de combinar enfocaments basats en biomarcadors amb noves immunoteràpies per desenvolupar estratègies més personalitzades per als pacients. La posada en marxa el 2025-2026 de l'assaig clínic ARI-HER2 reflecteix, a més, el potencial de traduir els avenços preclínic en beneficis per a les persones amb càncer de mama HER2-positiu.

Paraules claus: CAR-T, cancer de mama HER2-positiu, senyalització tònica.

INTRODUCTION

1. HER2-positive breast cancer

BC is the most common cancer worldwide in women, accounting for 11.5% of all tumor diagnoses in 2022, and remains one of the leading causes of cancer-related mortality in women^{1,2}. BC is a heterogeneous disease comprising four biological entities (Luminal A, Luminal B, Human epidermal growth factor receptor 2 [HER2, encoded by *ERBB2*] positive and triple negative) each defined by distinct molecular profiles, prognoses, and therapeutic targets. Although advances in local therapies such as surgery and radiotherapy, and systemic therapies including chemotherapy, endocrine, and targeted therapies have significantly improved survival and quality of life for many patients, approximately one-third of cases eventually relapse and progress to metastatic, incurable disease³.

HER2-positive BC represents approximately 15% of all BC cases and is characterized by the amplification of the HER2 gene and/or overexpression of the HER2 protein. This molecular alteration is historically associated with increased tumor aggressiveness, high proliferation rates, and poor prognosis compared to other subtypes. However, the introduction of HER2-targeted therapies—particularly trastuzumab, and more recently pertuzumab, T-DM1, and T-DXd—has radically transformed the natural history of the disease, leading to marked improvements in both early-stage and metastatic settings⁴. Despite its classification as a distinct subtype HER2-positive BC is biologically heterogeneous (**Figure 1**)³. A primary stratification is based on hormone receptor status, dividing tumors into hormone receptor-positive and hormone receptor-negative groups, which represent approximately two-thirds and one-third of HER2-positive cases, respectively. Nevertheless, hormone receptor status alone does not fully explain the diversity within HER2-positive disease. Gene expression profiling has allowed further subclassification into four intrinsic molecular subtypes that exhibit distinct patterns of gene activation, immune infiltration, response to treatment, and long-term prognosis: HER2-enriched (the most common, representing approximately 47% of cases), Luminal A (24%), Luminal B (20%), and Basal-like (around 9%)^{4,5}.

Importantly, approximately 5–10% of patients are diagnosed with HER2-positive

metastatic disease de novo, while a significant proportion of patients with initially localized HER2-positive BC—especially those with residual disease after neoadjuvant therapy or unfavorable molecular features—will eventually develop distant metastases⁶. Although the risk of metastatic relapse historically exceeded 30–40% in the pre-trastuzumab era, modern therapeutic strategies have reduced this risk substantially. Current data suggest that the 5-year risk of distant recurrence is now approximately 5–15%, depending on hormone receptor status, stage at diagnosis, and pathologic response to neoadjuvant treatment^{6,7}.

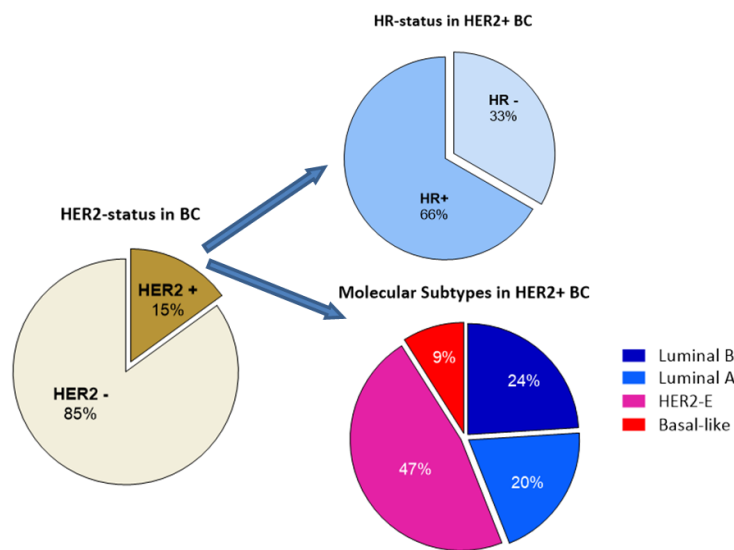


Figure 1. Biological and molecular heterogeneity in HER2-positive BC. Adapted from Agostinetto et al, 2024.

*HR: Hormone receptor

1.1 Treatment landscape of HER2-positive breast cancer

1.1.1 Early-stage breast cancer

Early-stage breast (EBC) cancer refers to BC that is confined to the breast and, in some cases, nearby lymph nodes, but has not spread to distant organs. The clinical management of EBC involves a comprehensive and multidisciplinary approach aimed at achieving curative outcomes, reducing the risk of recurrence, and preserving patients' quality of life. Treatment strategies are evolving toward personalized medicine, based not only on traditional clinical parameters such as tumor size and nodal involvement, but also on molecular subtype, histopathological features, and individual patient

characteristics including age, comorbidities, and personal circumstances.

In the context of HER2-positive early BC therapeutic advances over the past two decades have significantly improved patient outcomes. The backbone of treatment includes surgery, either breast-conserving surgery or mastectomy, followed by radiotherapy when indicated, particularly after breast-conserving surgery or in patients with positive lymph nodes⁸. Systemic therapy is a key component of care for HER2-positive early-stage BC. The current standard regimen combines trastuzumab and pertuzumab—monoclonal antibodies that target distinct extracellular domains of the HER2 receptor—with taxane-based chemotherapy. Trastuzumab, the first humanized anti-HER2 antibody, binds to extracellular domain IV of HER2, blocking downstream signaling pathways, inducing cell-cycle arrest, and triggering antibody-dependent cell-mediated cytotoxicity. Pertuzumab targets domain II, preventing HER2 dimerization with other HER receptors. Together, they provide a more complete blockade of HER2 signaling⁹.

For patients with lower-risk, node-negative disease, evidence from the APT¹⁰ and ATEMPT¹¹ trials shows that excellent outcomes can be achieved with less intensive adjuvant therapy, typically chemotherapy plus trastuzumab. In contrast, for patients with high-risk clinical features, such as node-positive disease or tumors above 2 cm in size, neoadjuvant dual HER2 blockade (trastuzumab and pertuzumab) and multi-agent chemotherapy is recommended. Trials including NeoSphere¹², TRYPHAENA¹³ and KRISTINE¹⁴, have demonstrated significant absolute increases in pathologic complete response (pCR) rates, a well-established surrogate marker of long-term prognosis¹⁵.

In the adjuvant setting, the addition of one year of pertuzumab to trastuzumab and chemotherapy provides a limited survival benefit, an absolute increase of 4.9% in iDFS and 1.8% in OS observed among patients with node-positive disease^{16,17} (**Figure 2**).

For patients who do not achieve a pCR after neoadjuvant therapy, treatment escalation with 14 cycles of T-DM1 significantly reduces recurrence risk compared with 14 cycles of trastuzumab, as shown in the KATHERINE trial¹⁸. This strategy enables risk-adapted treatment intensification based on response to initial therapy. Ongoing pivotal trials

such as DESTINY-Breast05 (NCT04622319) and COMPASSHER2-RD (NCT04457596) are evaluating novel agents including T-DXd and tucatinib in this setting.

For patients with hormone receptor positive and HER2-positive disease, endocrine therapy is recommended following completion of chemotherapy and anti-HER2 treatment. The therapeutic landscape for early HER2-positive BC is rapidly evolving, with efforts focused on optimizing sequencing, duration, and intensity of therapy to maximize cure rates while minimizing overtreatment and long-term toxicity. Incorporation of biomarkers and genomic assays, such as HER2DX, represents a promising step toward more individualized treatment strategies¹⁹.

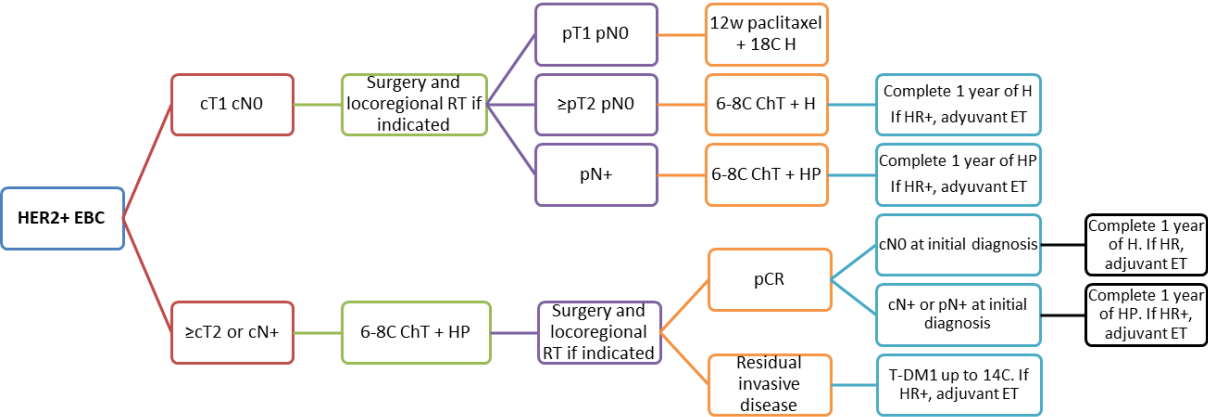


Figure 2. Treatment algorithm for patients with HER2-positive early-stage BC based on the European Society for Medical Oncology (ESMO) guidelines.* C: Cycles, ChT: Chemotherapy, EBC: early-stage breast cancer, ET: Endocrine Therapy, H: Trastuzumab, HR+: Hormone Receptor Positive, P: Pertuzumab, RT: Radiotherapy, W: weeks

1.1.2 Metastatic breast cancer

Although this stage of the disease is considered incurable, recent therapeutic advances have significantly improved its manageability. Defining an optimal treatment strategy for patients with HER2-positive metastatic breast cancer (mBC) remains a critical clinical goal. This imperative stems from both the biological aggressiveness of HER2-driven tumors and the rapidly evolving therapeutic landscape, which offers an expanding arsenal of agents with distinct mechanisms of action. The increasing complexity of treatment options requires a personalized approach, one that considers not only tumor biology—such as hormone receptor status, intrinsic molecular subtype,

and presence of central nervous system (CNS) involvement—but also prior treatments, disease burden, and patient-specific clinical factors.

To date, three therapeutic strategies dominate the standard of care in the first three lines of mBC treatment (**Figure 3**), each targeting the HER2 receptor through different modalities:

- **Dual HER2 blockade with chemotherapy**

The standard first-line regimen is based on the combination of trastuzumab and pertuzumab. The approval of pertuzumab is based on the results of the CLEOPATRA trial⁷. This phase III studied the efficacy of trastuzumab and docetaxel with pertuzumab or placebo as first-line treatment in 808 patients with HER2-positive mBC. The results of CLEOPATRA revealed that patients who received pertuzumab had significantly longer progression-free survival (PFS) than those who received placebo (median 18.7 v 12.4 months, hazard ratio [HR] of 0.62, $P < .001$). Overall response rate (ORR) was 69.3% in the control group, compared with 80.2% in the pertuzumab group⁷. Of note, complete response in the pertuzumab arm was achieved in 5.5% of patients. After approximately 100 months of follow-up, overall survival (OS) was significantly longer with pertuzumab than placebo (HR, 0.69; $P < .001$), with a median OS of 57.1 versus 40.8 months²⁰. Dual blockade with trastuzumab and pertuzumab was approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for first-line therapy for patients with de novo metastatic HER2-positive BC or patients relapsing after 1 year of ending an anti-HER2 adjuvant treatment.

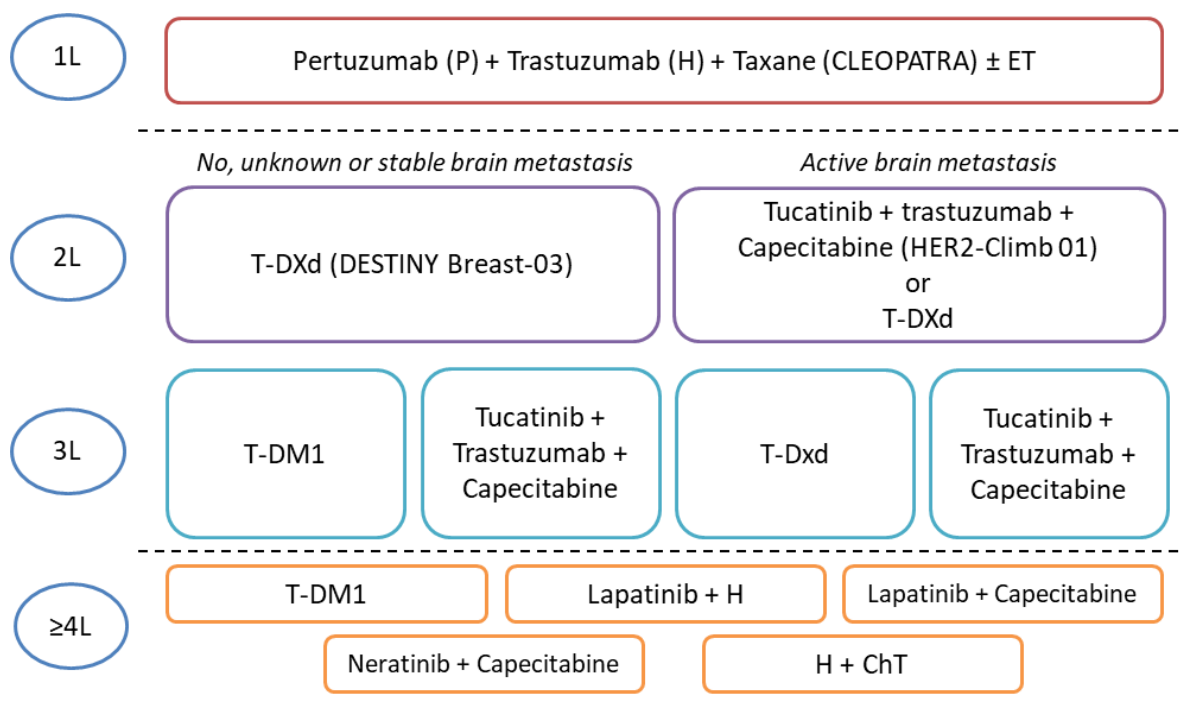


Figure 3. Treatment algorithm for patients with HER2-positive mBC based on the ESMO guidelines. *
ChT: Chemotherapy, ET: Endocrine Therapy, H: Trastuzumab, L: Line.

▪ Antibody–drug conjugates

Upon progression to dual HER2 blockade with chemotherapy, the preferred treatment is an antibody–drug conjugate (ADCs). ADCs were designed to channel the cytotoxic effects of chemotherapy to specific tumor cells. ADCs contain a tumor-targeting antibody covalently bound to a cytotoxic drug (payload) via a synthetic linker²¹ The ADC is directed to cancer cells expressing the target (for example, HER2) on the cell surface, followed by internalization of the ADC and release of the cytotoxic payload, resulting in tumor cell death²².

T-DXd, a second-generation ADC, has largely replaced T-DM1 as the second-line standard, owing to the results of the DESTINY-Breast03 trial²³. In this phase III trial, T-DXd demonstrated clear superiority over T-DM1. The median PFS was 28.8 months (95% CI, 22.4–37.9) for T-DXd compared to 6.8 months (95% CI, 5.6–8.2) for T-DM1, representing a 67% reduction in the risk of disease progression or death (HR 0.33). Regarding OS, the median was 52.6 versus 42.7 months (HR, 0.73; 95% CI, 0.56–0.94), respectively²⁴. The objective response rate (ORR) was also

significantly higher with T-DXd, reaching 79.7% versus 34.2% with T-DM1. Additionally, T-DXd showed promising intracranial activity in patients with stable brain metastasis. In terms of safety, grade 3 or higher adverse events were somewhat more frequent with T-DXd (52.6%) than with T-DM1 (48.4%). The most common side effects associated with T-DXd included nausea, fatigue, vomiting, and alopecia. One of the most notable adverse events was interstitial lung disease or pneumonitis, reported in approximately 10% of patients treated with T-DXd, mostly mild to moderate in severity, with no fatal cases reported in this trial.

T-DXd was even more efficacious than the combination of trastuzumab or lapatinib with capecitabine, with a median PFS of 17.8 vs. 6.9 months and median OS of 39.2 vs. 26.5 months, respectively²⁵.

- **HER2-targeted tyrosine kinase inhibitors**

Tyrosine kinase inhibitors are small molecules that target the intracellular catalytic kinase domain of HER2, competing with ATP, blocking phosphorylation and activating downstream signaling cascades²². For patients progressing after T-DXd—or those with CNS involvement—the combination of tucatinib, trastuzumab, and capecitabine has emerged as a preferred option. The HER2CLIMB trial²⁶ demonstrated that this triplet significantly improved both systemic and CNS disease control. In the overall study population, median PFS was 7.6 months vs. 4.9 months, and median OS was 24.7 vs. 19.2 months, compared to treatment with trastuzumab plus capecitabine alone. Remarkably, in patients with active brain metastases, median OS reached 18.1 months with the triplet regimen, representing a substantial advance in a historically challenging subgroup. Other tyrosine kinase inhibitors, such as lapatinib and neratinib, are used beyond the third line, providing additional options in the setting of advanced disease.

Despite the efficacy of these agents, key challenges remain in later-line treatment. The optimal sequencing of HER2-targeted ADCs, especially those sharing the same epitope (e.g., HER2), is not yet well defined. The clinical benefit of T-DM1 following T-DXd is unclear, and cross-resistance may reduce its efficacy. A real-world retrospective study

conducted in France suggested that tucatinib-based therapy retains some activity after T-DXd, but outcomes were less favorable than those seen in HER2CLIMB, with median PFS of 4.7 months and OS of 13 months²⁷. Additionally, re-challenging patients with dual HER2 blockade using trastuzumab and pertuzumab combined with chemotherapy is no longer recommended. The PRECIOUS trial²⁸, conducted in Japan, failed to demonstrate a meaningful PFS benefit for this regimen compared with chemotherapy plus trastuzumab alone. Moreover, imbalances between treatment arms in patient characteristics and chemotherapy backbones limited the interpretation of the reported OS advantage.

1.1.3 Progress and future directions in advanced HER2-positive breast cancer treatment

The current treatment algorithm for advanced HER2-positive BC is expected to evolve significantly as results from several pivotal ongoing clinical trials become available. The phase III DESTINY-Breast09 trial (NCT 04784715) is currently evaluating T-DXd, with or without pertuzumab, compared to the CLEOPATRA regimen as first-line therapy in patients with previously untreated advanced HER2-positive disease. Given the remarkable outcomes of the DESTINY-Breast03 trial, in which T-DXd demonstrated a PFS in the second-line setting that exceeded that reported for first-line therapy in CLEOPATRA, it is highly plausible that T-DXd could soon replace the current standard and be adopted as the preferred first-line treatment.

In the second-line setting, both T-DXd monotherapy and the triplet regimen of tucatinib, trastuzumab, and capecitabine are being reevaluated. The HER2CLIMB-04 trial HER2CLIMB-04 trial²⁹ is exploring the potential synergy of combining T-DXd with tucatinib, while additional studies are assessing alternative strategies: T-DM1 plus tucatinib in the HER2CLIMB-02 trial (NCT03975647), and T-DM1 plus atezolizumab in the KATE3 study³⁰.

Another emerging approach involves bispecific antibodies targeting HER2. For example, KN026, a bispecific antibody binding to domains II and IV of HER2, showed encouraging

results when combined with docetaxel as first-line treatment in HER2-positive mBC, achieving an overall response rate (ORR) of 76.4% and a median PFS of 27.7 months³¹. However, direct comparisons with the established trastuzumab–pertuzumab backbone remain lacking.

Moreover, several agents are being tested in combination with dual HER2 blockade (trastuzumab and pertuzumab) as maintenance therapy in the first-line setting. These include: atezolizumab (NRG-BR004³²), tucatinib (HER2CLIMB-05, NCT05132582), palbociclib (PATINA, in ER-positive tumors³³), giredestrant (HeredERA, in ER-positive tumors³⁴), and alpelisib (EPIK-B2, NCT04208178, in PIK3CA-mutated tumors).

HER3 has recently emerged as a relevant therapeutic target. Zenocutuzumab (MCLA-128), a bispecific antibody against HER2 and HER3, demonstrated modest activity in heavily pretreated HER2-positive mBC, achieving an ORR of 27% and a 24-week clinical benefit rate of 49% in a phase II study³⁵. Seribantumab (MM-121), a monoclonal antibody directed against HER3, has shown the ability to suppress tumor growth in NRG1 fusion-driven models and was evaluated in a phase II trial (NCT04383210) across advanced solid tumors, with early results suggesting durable responses and favorable tolerability³⁶.

The most promising results, however, come from antibody–drug conjugates (ADCs) targeting HER3. Patritumab deruxtecan (HER3-DXd) combines an anti-HER3 antibody with deruxtecan (DXd) via a cleavable linker. In a phase I/II study of heavily pretreated mBC (median of five prior lines), HER3-DXd demonstrated notable antitumor activity across all BC subtypes, with a 43% response rate and median PFS of 11 months in HER2-positive, HER3-expressing tumors (n=14)³⁷. Based on these findings, HER3-DXd has also been investigated in HER2-negative disease as exemplified by the SOLTI-TOT-HER3 trial^{38,39}.

Collectively, these trials may redefine the sequencing and selection of targeted therapies in HER2-positive mBC⁴.

1.2 Mechanisms of resistance to HER2 therapy

Resistance to HER2-targeted therapies can develop through diverse mechanisms that vary depending on the therapeutic approach employed. In particular, treatments such as trastuzumab and ADCs like T-DM1 and T-DXd are associated with multifactorial resistance pathways. These include HER2 intratumoral heterogeneity, alterations in HER2 expression or mutations, dysregulation of proteins involved in the HER2 signaling pathway, impaired internalization of the HER2-drug complex, lysosomal degradation issues and resistance to the cytotoxic components of T-DM1 and T-DXd, among others. This subset—comprising over 20% of patients with HER2-positive BC—represents a significant unmet clinical need, underscoring the importance of developing novel therapeutic strategies^{40–42}.

1.2.1 HER2 intratumoral heterogeneity

HER2 intratumoral heterogeneity refers to the variation in HER2 expression and distribution among different subpopulations of cells within a tumor. This heterogeneity can lead to varying degrees of dependence on HER2 signaling as an oncogenic driver, resulting in differential sensitivity to HER2-targeted therapies⁴³ (**Figure 4 A**).

In an analysis of the MARIANNE trial—which did not demonstrate the superiority of T-DM1 over a combination of taxane and trastuzumab—patients whose tumors exhibited heterogeneous HER2 expression, as assessed by immunohistochemistry, experienced a numerically shorter median PFS compared to those with homogeneous expression⁴⁴. Similar findings have been reported in studies of early-stage HER2-positive BC, where HER2 heterogeneity has emerged as a potential biomarker associated with suboptimal response and poorer outcomes following systemic HER2-targeted therapy^{45–47}.

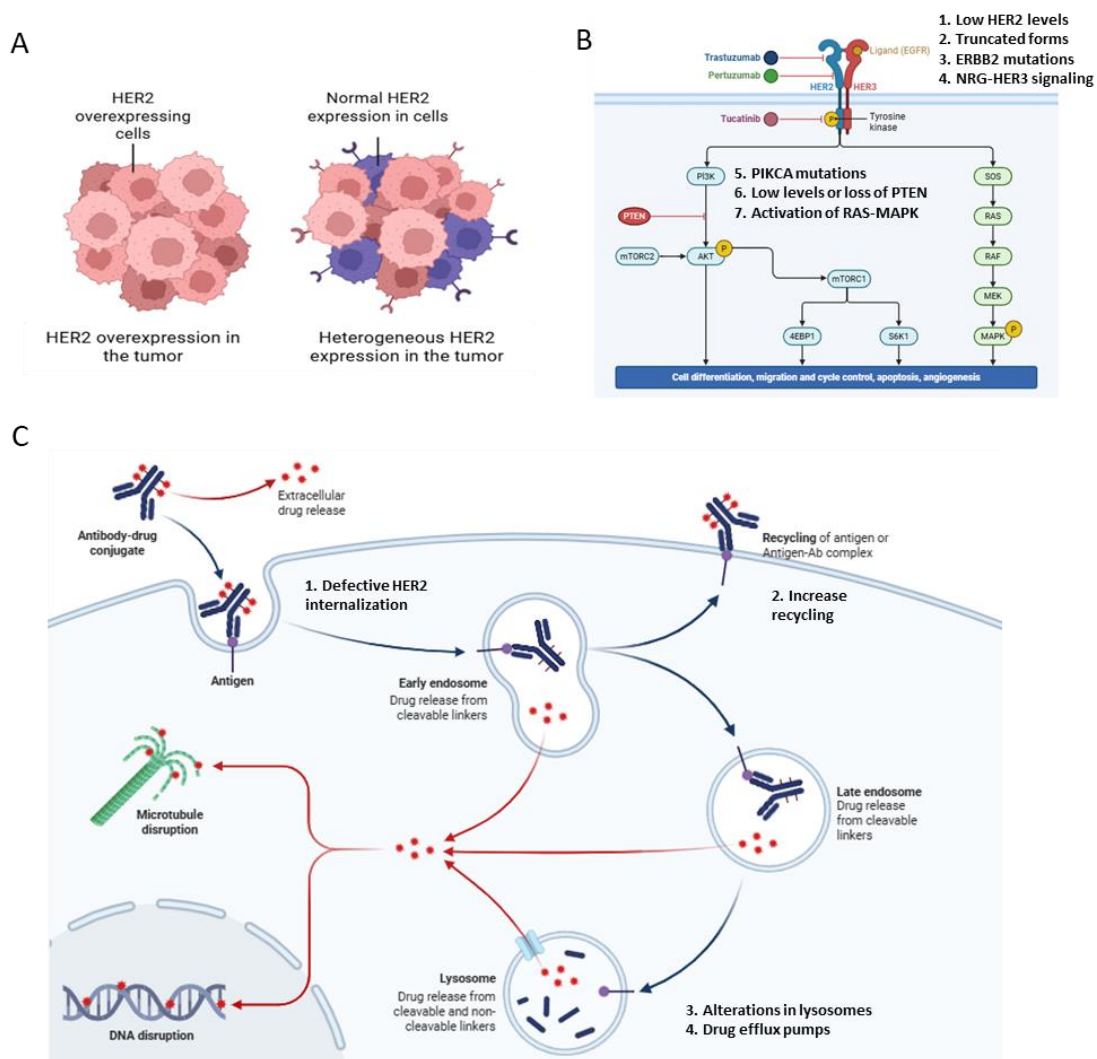


Figure 4. Mechanisms of resistance to HER2-targeted therapies. A. Heterogeneous expression of the HER2 receptor within tumors reduces the overall efficacy of HER2-targeted treatments. B. Mutations or alterations in HER family receptors, along with the activation of compensatory signaling pathways, can sustain downstream signaling despite HER2 inhibition. C. Disruptions in HER2 internalization, recycling processes, lysosomes degradation and drug efflux pumps can impair therapeutic effectiveness of ADC. Created with biorender.

1.2.2 HER2 loss and pathway dysregulation

Loss of HER2 expression is not uncommon, occurring in up to 50% of breast tumors across various clinical settings, and has been associated with poorer recurrence-free survival. Multiple mechanisms may underlie this phenomenon, including intratumoral heterogeneity, clonal selection, and genuine biological alterations such as HER2 downregulation or molecular subtype switching^{48,49}. Additionally, the presence of p95HER2—a truncated form of the HER2 oncoprotein—has been linked to unfavorable outcomes⁵⁰. Emerging evidence also indicates that the frequency of *ERBB2* mutations in HER2-positive BC increases with the number of prior lines of HER2-directed treatment, highlighting the dynamic evolution of resistance under therapeutic pressure⁵¹ (**Figure 4 B**).

Furthermore, activation of alternative downstream intracellular signaling pathways that drive tumor progression despite HER2 blockade has been associated with resistance to HER2-targeted therapies. In most cases, this resistance arises from compensatory mechanisms that reactivate key HER2 downstream pathways, particularly PI3K–AKT and RAS–MAPK⁵².

Additionally, activation of alternative receptor tyrosine kinases, particularly via HER3 ligands like neuregulin-1, can trigger compensatory signaling pathways (e.g., PI3K/AKT), diminishing T-DM1 sensitivity⁵³. These mechanisms often correlate with decreased HER2 expression and increased EGFR and integrin levels, contributing to more aggressive phenotypes and therapeutic resistance⁵⁴.

1.2.3 Alterations in the internalization of HER2

Once ADCs bind to HER2 on the cell surface, the complex is internalized into endosomes, a process that can be facilitated by the endocytic protein endophilin A2. Notably, silencing of endophilin A2 in HER2-positive cells has been shown to impair HER2 internalization and reduce sensitivity to trastuzumab and T-DM1⁵⁵. Additionally, the processes of HER2 ubiquitination and trafficking to lysosomes may be disrupted by its constitutive association with the chaperone HSP90, thereby compromising the efficacy of HER2-targeted therapies⁵⁶ (**Figure 4 C**).

Moreover, T-DM1 may be internalized via caveolae (membrane structures composed of caveolin-1), which diverts the ADC from lysosomal degradation, ultimately diminishing its therapeutic efficacy⁵⁷ and contributing to drug resistance.

Finally, increased recycling of HER2-containing endosomes back to the plasma membrane may limit the delivery of ADCs to lysosomes, reducing their cytotoxic potential⁵⁸. HER2 is known for its rapid recycling kinetics, which can contribute to this effect.

1.2.4 Alterations in lysosomes and efflux pumps

The trafficking of ADCs to lysosomes and their subsequent processing by lysosomal enzymes are critical steps for the effective release of the cytotoxic payloads linked to the antibody. Lysosomes are acidic organelles, and their proteolytic activity relies on the vacuolar H⁺-ATPase, a proton pump responsible for maintaining the low pH required for optimal enzyme function. Several studies have reported that increased lysosomal pH and decreased lysosomal enzyme activity contribute to resistance to T-DM1⁵⁹ (**Figure 4 C**).

Resistance to T-DM1 can arise through increased expression or activity of ABC-family drug efflux pumps, which export the cytotoxic catabolite Lys-MCC-DM1 and reduce its intracellular efficacy⁶⁰.

1.3 Prognostic and predictive biomarkers in HER2-positive disease

Considering the wide range of available HER2-targeted therapies, having tools to guide the optimal sequencing of these treatments would be highly valuable. However, the ideal sequence remains unknown and likely depends on several factors, including prior adjuvant therapies, the length of the relapse-free interval, the efficacy of previous treatments in the advanced setting, tumor burden, patient preferences, quality of life, as well as drug availability and cost in each country. Currently, no biomarkers are available to guide therapy response, highlighting an

urgent need to identify which patients will benefit from specific therapies and which may require other therapeutic strategies⁶¹.

1.3.1 HER2DX: new tool for clinical management of HER2-positive disease

Recently, the Translational Genomics and Targeted Therapies in Solid Tumors group at IDIBAPS, of which I am a member, developed the HER2DX assay, the first integrated prognostic tool combining clinicopathological and genomic variables for early-stage HER2-positive BC¹⁹. HER2DX provides two independent scores: one predicting long-term prognosis (i.e., risk score) and the other estimating the likelihood of achieving a pCR following trastuzumab-based neoadjuvant therapy (i.e., pCR score). This assay identifies a substantial subset of patients with favorable outcomes who may not require additional therapies, such as pertuzumab, neratinib, or trastuzumab emtansine, beyond chemotherapy and trastuzumab (with endocrine therapy if hormone receptor-positive). Conversely, HER2DX can also identify high-risk patients who may benefit from treatment escalation with additional anti-HER2 agents. Thus, HER2DX represents a valuable tool for guiding both de-escalation and escalation strategies in early-stage HER2-positive BC.

The HER2DX scores (i.e., risk score and pCR likelihood score) are derived from four distinct gene expression signatures encompassing 27 genes, which reflect key biological processes including immune infiltration, luminal differentiation, tumor cell proliferation, and HER2 amplicon expression^{19,62}. These genomic signatures are integrated with clinical variables such as tumor size and nodal status to generate individualized predictions. Notably, each component of the HER2DX model is differentially associated with treatment response in the neoadjuvant setting and with long-term prognosis. Among them, the IGG signature is the only feature within HER2DX that correlates with both improved response to neoadjuvant anti-HER2 therapy and enhanced survival outcomes (**Figure 5**). This IGG signature was originally identified through unsupervised clustering of breast tumors and has demonstrated strong independent prognostic value in a large cohort (n=550)⁶³ of patients who did not receive systemic adjuvant therapy and has been externally validated across diverse

clinical cohorts involving over 1,000 patients^{19,64}. The IGG comprises genes involved in lymphocyte progenitor maturation, activation, differentiation, immunoglobulin production, chemotaxis, and lymphocyte activity regulation. Understanding the role of the IGG signature in modulating treatment response is therefore of critical importance⁶⁵.

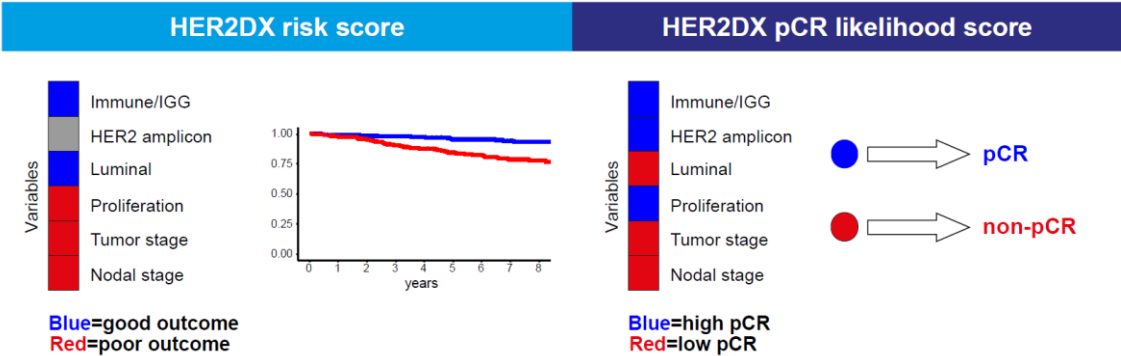


Figure 5. Summary of the variables included in the HER2DX assay and their association with each clinical endpoint. The type of association between a variable and each clinical endpoint is represented in different colors, where red means that a high score of that variable is associated with worse survival outcome or a lower likelihood of achieving a pCR, and blue means that a high score of that variable is associated with better survival outcome or a higher likelihood of achieving a pCR. Grey means no association of the variable with the clinical endpoint. From Prat et al, Ebiomedicine 2022.

1.3.2 *ERBB2* mRNA score

The HER2DX assay quantifies *ERBB2* mRNA as part of a validated multigene signature and provides a robust measure of tumor HER2 transcriptional abundance¹⁹. Unlike conventional immunohistochemistry (IHC) or in situ hybridization (FISH), *ERBB2* mRNA measurement captures the full spectrum of transcriptional heterogeneity observed in HER2-positive BC¹⁹. In early-stage disease, higher *ERBB2* mRNA levels correlate with an increased likelihood of achieving pCR after neoadjuvant anti-HER2-based therapy and with improved event-free and overall survival when analyzed both as a continuous variable and within pre-specified *ERBB2*-low, -medium, and -high categories. These findings position the *ERBB2* mRNA score as a complementary biomarker to standard HER2 testing⁶⁶.

Importantly, *ERBB2* mRNA provides clinically actionable information beyond the main biological drivers captured by intrinsic subtyping. On one hand, *ERBB2* mRNA levels have been shown to accurately predict clinical HER2 status as defined by ASCO/CAP guidelines. On the other hand, they are strongly associated with therapeutic response across settings, including neoadjuvant regimens and antibody–drug conjugates. In the KATHERINE phase III trial, residual tumors with higher *ERBB2* mRNA expression derived greater benefit from adjuvant T-DM1 compared with trastuzumab, supporting its predictive utility in the post-neoadjuvant setting⁶⁷.

Further evidence links *ERBB2* mRNA expression with treatment sensitivity to T-DM1⁶⁸ and dual HER2 blockade⁶⁹. A HER2DX-based analysis of patients receiving T-DM1 demonstrated a clear gradient of clinical benefit: objective response rates were 0% in *ERBB2*-low tumors, 29% in *ERBB2*-medium, and 56% in *ERBB2*-high tumors. Similarly, higher *ERBB2* mRNA expression correlated with longer PFS and OS⁶⁸.

In the advanced/metastatic setting, a recent study confirmed that *ERBB2* mRNA retains prognostic value in patients treated with first-line chemotherapy plus trastuzumab and pertuzumab (THP), potentially enabling stratification of those most likely to benefit from standard dual HER2 blockade⁷⁰.

Collectively, these data establish the HER2DX *ERBB2* mRNA score as both a prognostic biomarker, identifying patients with more favorable outcomes, and a predictive biomarker, indicating likelihood of response to anti-HER2 therapies.

1.3.3 Tumor-infiltrating lymphocytes (TILs)

Several variables beyond tumor burden have been associated with patients' prognosis and/or treatment response in early-stage BC. Among these, hormone receptor status and the intrinsic molecular subtypes (Luminal A, Luminal B, HER2-enriched, and Basal-like) play a significant role in influencing therapeutic outcomes and survival rates.

Another well-established prognostic and predictive factor is the percentage of stromal TILs, which reflect the host immune response within the tumor microenvironment (TME)^{71,72}.

TILs prevalence is highly heterogeneous among BC subtypes. Triple-negative breast cancer (TNBC) exhibits the highest levels of immune infiltration, followed by HER2-positive, and then Luminal subtypes, which typically display the lowest TIL content^{73,74}. Specifically, high TILs levels have been associated with improved pCR rates after neoadjuvant chemotherapy across subtypes. In early-stage HER2-positive BC, elevated stromal TILs correlate with significantly higher pCR rates and are consistently associated with favorable outcomes in the context of HER2-targeted therapy^{73,75–78}. For instance, studies have reported that HER2-positive patients with high TILs levels (>30%) achieve pCR rates approaching 60–70% following dual HER2 blockade with trastuzumab and pertuzumab^{77,79–83}.

Beyond stromal TILs quantification, detailed immune characterization is becoming increasingly relevant. The T cell-mediated antitumor response has been a key area of focus, particularly regarding cytotoxic CD8+ TILs. The presence of tumor-infiltrating T lymphocytes (TIL-T) cells has been shown to enhance the efficacy of anti-HER2 therapies⁸⁴. The role of B-cell-mediated response to therapy has been less explored. Emerging evidence supports the prognostic value of tumor-infiltrating B lymphocytes (TIL-B), especially those organized in TLS. TLS are ectopic lymphoid aggregates that resemble secondary lymphoid organs and support local antigen presentation and immune activation⁸⁵. TIL-B within TLS have been associated with favorable prognosis in HER2-positive BC⁸⁶ and may function as antigen-presenting cells that prime anti-tumor T cell responses^{87,88}. Recent spatial transcriptomics studies have confirmed the co-localization of B and T cells within TLS, suggesting their functional synergy in shaping anti-tumor immunity⁸⁹.

T- and B-cell receptor diversity is another critical factor that may determine disease progression and response to therapy. T-cell receptors (TCRs) and B-cell receptors (BCRs) are generated through V(D)J recombination—a stochastic recombination of variable

(V), diversity (D), and joining (J) gene segments—yielding vast antigen receptor repertoires capable of recognizing a wide array of tumor-associated antigens. In addition, BCRs can also undergo subsequent somatic hypermutation (SHM), and class switch recombination⁹⁰. These processes create a functionally diverse and dynamic set of T- and B-cells. The estimated combinatorial diversity of TCRs and BCRs ranges from 10^{12} to 10^{18} unique sequences, underlining the complexity and adaptability of the immune repertoire⁹¹.

The diversity of the TCR of TIL-T has shown prognostic values in various solid tumors, including BC⁹². High BCR sequence variants have been reported in basal-like and HER2-enriched subtypes, consistent with SHM and a tumor antigen-driven response⁹³. Moreover, TIL-B within breast tumors exhibit higher clonality than B-cells in peripheral blood and are significantly associated with improved patient survival⁹⁰. Further studies are needed to better understand the diversity of TCR and BCR and their impact on response to therapies.

1.3.4 Other biomarkers

Moreover, additional biomarkers with potential clinical utility have been evaluated. These include PD-L1 expression assessed by immunohistochemistry, which has emerged as a predictor of response to immune checkpoint inhibitors^{94,95}, and PIK3CA somatic mutations which are associated with poorer responses to anti-HER2 therapies but may predict benefit from alfa-specific PI3K inhibitors⁹⁶.

Beyond these molecular markers, genomic tools such as circulating tumor DNA (ctDNA) analysis are under active investigation. Plasma ctDNA levels hold promise not only as a dynamic biomarker for monitoring disease burden and treatment efficacy but also as a potential guide to address unresolved clinical questions—such as the optimal duration of anti-HER2 maintenance therapy in patients who remain without progression for long periods⁶¹.

2 Immuno-oncology, a new era to fight cancer

Immunotherapy has fundamentally transformed the landscape of cancer treatment,

bringing new hope to patients with both solid and hematologic malignancies who previously had limited therapeutic options. Among the diverse immunotherapeutic approaches, adoptive cell therapy (ACT) has emerged as one of the most promising and revolutionary strategies.

ACT is a personalized treatment in which a patient's immune cells are collected, expanded or genetically engineered in the laboratory, and then reinfused to enhance the immune system's ability to recognize and eliminate cancer cells.

The first clinical application in solid tumors of ACT involved TILs, particularly in metastatic melanoma⁹⁷. These lymphocytes, isolated directly from the TME, demonstrated intrinsic tumor-recognition capabilities. Their ex vivo expansion using interleukin-2 (IL-2) followed by reinfusion into the subject led to durable clinical responses in a subset of patients, marking a milestone in the evolution of cancer immunotherapy (**Figure 6 A**). Since the pioneering studies conducted by Rosenberg and collaborators⁹⁸, numerous phase I/II trials from different institutions have proved the efficacy of unselected TIL therapy in patients with advanced cutaneous melanoma, with objective response rates around 40-50% and a significant proportion of sustained complete responses^{99,100,101,102,103,104}. In 2022, the first phase III trial evaluating TIL therapy demonstrated its superiority over the anti-CTLA-4 antibody ipilimumab in patients with metastatic melanoma (most of whom were refractory to anti-PD1 therapy), reporting an objective response rate (ORR) of 49% (vs 21%) and a 20% of complete responses¹⁰⁵. Based on this accumulated evidence, FDA approved the autologous TIL therapy lifileucel in February 2024 for patients with immune checkpoint inhibitor-refractory metastatic melanoma, making it the first cell therapy approved for solid tumors¹⁰⁶.

A key prerequisite for TIL-based therapy is the presence of tumor-reactive T cells within the patient's immune system, which must be isolated and expanded from resected tumors. While TIL therapy has shown notable success in melanoma, its application to other cancer types has been more limited and less effective.

To address the limitations of TIL therapy, T cells can be genetically engineered to express tumor-specific receptors—either TCRs or CARs—allowing them to recognize and attack cancer cells that would otherwise evade immune detection. This strategy enables the conversion of any T cell into an anti-tumor effector cell, significantly expanding the potential of ACT to a broader range of solid tumors. Both TCR- and CAR-T cell therapies follow a similar process: peripheral blood T cells are isolated, genetically modified to express tumor-specific receptors, expanded in vitro, and reinfused into the patient after lymphodepletion¹⁰⁷ (**Figures 6 B-C**).

TCR therapy represents another sophisticated form of adoptive cell therapy. Unlike CAR-T cells, which recognize only surface antigens, TCR-T cells can target intracellular proteins presented on the cell surface by MHC molecules, thereby expanding the range of potential targets. In 2024, the FDA approved afamitresgene autoleucel (afami-cel, Tecelra) for metastatic synovial sarcoma¹⁰⁸. In pivotal clinical trials, over 40% of patients experienced tumor reduction, with a median duration of response of six months.

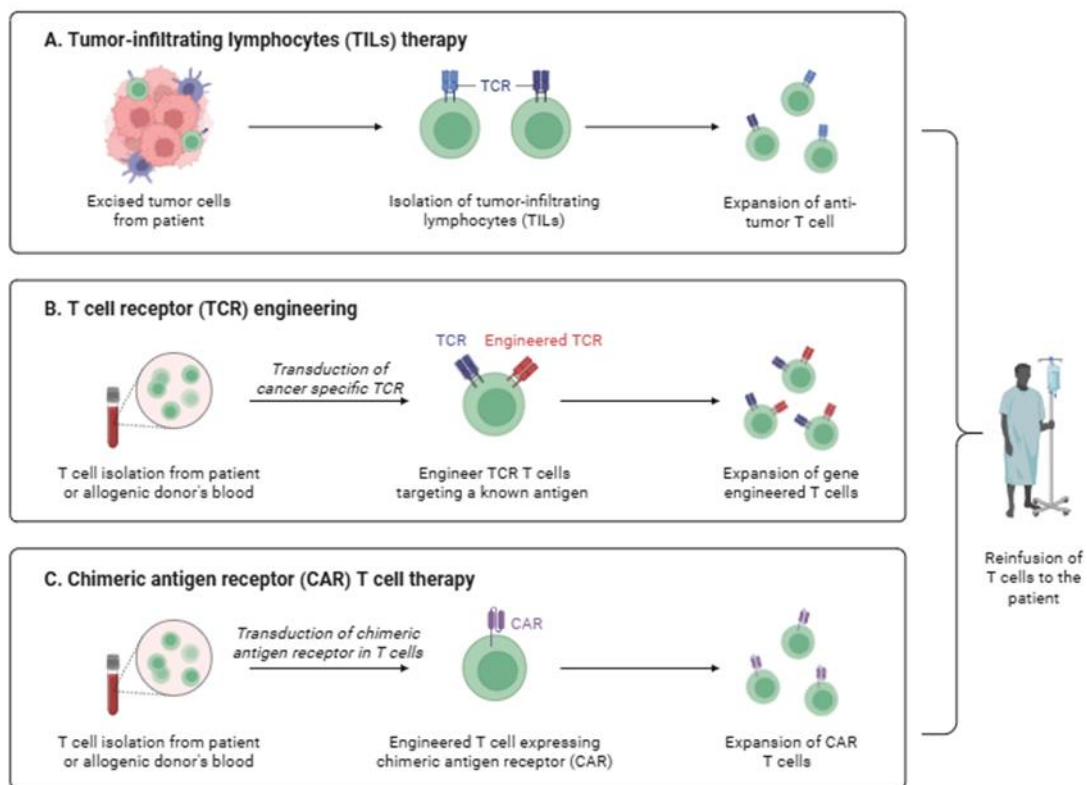


Figure 6. Ex vivo expansion process for adoptive cell therapy. The figure represents ex vivo expansion workflows for TILs (A), TCR-T (B), and CAR T cells (C). Created by biorender.

2.1 CAR-T cell therapy

CAR-T therapy has revolutionized the treatment of hematologic cancers. This approach involves the genetic modification of a patient's T cells to express synthetic receptors that recognize specific surface antigens on cancer cells, independent of MHC presentation¹⁰⁹. In addition to T cells, CARs can also be used in other immune cells, such as macrophages and natural killer (NK) cells.

CAR-T therapy has demonstrated outstanding efficacy in treating B-cell malignancies, including acute lymphoblastic leukemia, diffuse large B-cell lymphoma, and multiple myeloma. As of 2025, seven FDA-approved CAR-T products—Kymriah, Yescarta, Tecartus, Breyanzi, Abecma, Carvykti, and Aucatzyl—are available, each designed to target specific hematologic cancers¹¹⁰.

However, the application of CAR-T therapy to solid tumors presents a new set of challenges due to the complexity of the TME, antigen heterogeneity, and physical barriers to T cell infiltration. Overcoming these obstacles represents the next frontier- and opportunity-for innovation in this field.

2.1.1 CAR design

CARs are synthetic constructs that provide T cells with the ability to recognize and kill tumor cells in an MHC-independent manner¹⁰⁹. These engineered receptors combine antigen-binding and intracellular signaling functionalities into a single molecule. Once introduced into T cells, the modified cells acquire potent effector capabilities, often described as "living drugs" due to their capacity for both immediate cytotoxicity and long-term persistence¹¹¹.

Structurally, CARs are composed of four modular domains: an extracellular antigen-binding region, a hinge or spacer domain, a transmembrane domain, and an intracellular signaling domain. The extracellular domain is most commonly a single-chain variable fragment (scFv), derived from monoclonal antibodies and predominantly composed of the variable light and heavy chain regions separated by a flexible linker.

This fragment is responsible for recognizing a specific tumor-associated antigen. Its properties—such as affinity, avidity, epitope location, and propensity for aggregation—play a central role in determining the CAR's specificity, activation threshold, and overall functionality. While scFvs remain the most widely used recognition elements, alternative binding domains such as nanobodies, ligands for cognate receptors, native receptor regions, and even small peptides have also been explored^{112,113} (**Figure 7**).

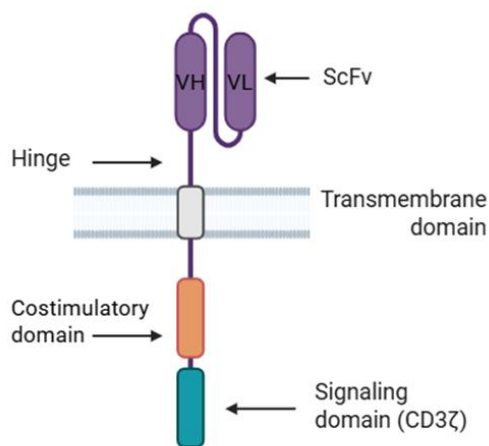


Figure 7. CAR prototype. The extracellular domain typically originates from the single-chain variable fragment (scFv) of a monoclonal antibody. The hinge region provides flexibility and serves as a spacer between the scFv and the cell membrane. The transmembrane domain secures the receptor within the membrane, while the intracellular portion comprises a costimulatory domain and the CD3 ζ chain, which together initiate signal transduction upon antigen recognition. Created with biorender.

Following the scFV, the hinge or spacer domain connects the binding region to the transmembrane segment, providing structural flexibility and ensuring appropriate positioning of the scFv above the T cell surface. This domain is essential for effective antigen recognition, particularly when the epitope is located close to or far from the tumor cell membrane. Commonly, the hinge is derived from the CD8 or CD28 sequences, selected in coordination with the transmembrane domain to maintain structural compatibility. The transmembrane domain anchors the CAR within the T cell membrane and contributes to receptor stability and surface expression. Like the hinge, it is typically sourced from transmembrane proteins such as CD8 α or CD28^{112,113}.

The intracellular region of the CAR is responsible for signal transduction and T cell activation. All CARs contain the CD3 ζ signaling chain, which includes immunoreceptor tyrosine-based activation motifs (ITAMs) essential for initiating T cell responses, as well as one or more costimulatory domains.

The most extensively studied co-stimulatory domains in CAR-T cell therapy are those derived from CD28 or 4-1BB (CD137), both of which are incorporated into all FDA-approved CAR-T cell products to date. Although CAR-T cells demonstrate high response rates across patients, CD28 and 4-1BB domains confer distinct functional and metabolic characteristics^{114,115}. CARs incorporating CD28 domains activate signaling through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, promoting enhanced IL-2 production, differentiation into effector memory T-cells, and a metabolic shift toward aerobic glycolysis. In contrast, CAR-T cells containing 4-1BB domains engage TNF receptor-associated factor (TRAF) proteins, differentiate into central memory T-cells, and exhibit increased mitochondrial biogenesis and reliance on oxidative metabolism^{116,117}.

Over the years, CARs have evolved through successive generations, based on the number of costimulatory domains, each incorporating structural and functional enhancements to improve antitumor efficacy, persistence, and safety (**Figure 8**). First-generation CARs consist of a single CD3 ζ signaling domain, which provides the primary activation signal upon antigen recognition. However, these CARs showed limited clinical success due to insufficient T cell proliferation and persistence. To address this, second-generation CARs added a single co-stimulatory domain (CD28, 4-1BB, ICOS or OX40) to the intracellular region, thereby enhancing T cell expansion, survival, and cytokine production. Third-generation CARs further improved on this design by incorporating two co-stimulatory domains aiming to synergize distinct signaling pathways and maximize therapeutic activity. Finally, fourth-generation CARs, also known as TRUCKs (T cells redirected for antigen-unrestricted cytokine-initiated killing), build on the second-generation backbone but include additional genetic elements such as inducible cytokine genes or safety switches. Some researchers have proposed a fifth generation of CARs, characterized by the incorporation of a truncated cytoplasmic domain from the IL-2 receptor along with a STAT3-binding motif. These additions allow for antigen-dependent activation of the JAK-STAT signaling cascade, particularly STAT3 and STAT5, thereby promoting a more physiologic and sustained T cell response^{109,118}.

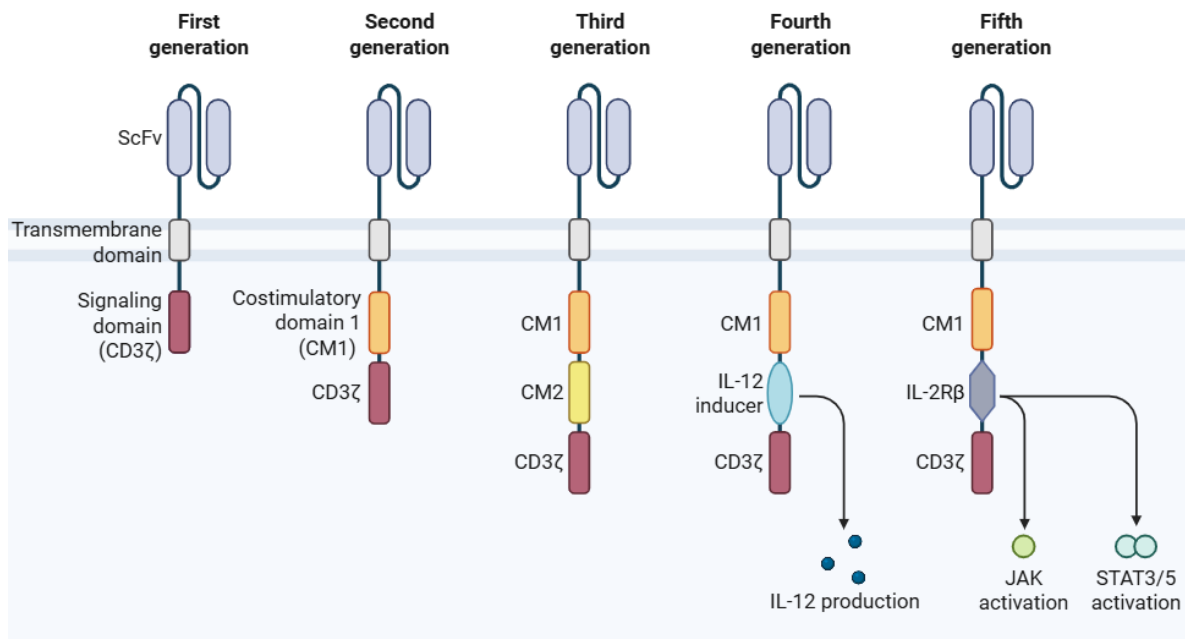


Figure 8. Evolution of chimeric antigen receptor (CAR) designs. First-generation CARs are composed of an extracellular antigen-binding domain linked to the CD3ζ signaling domain, providing a basic activation signal. Second-generation CARs enhance T cell function by incorporating a single co-stimulatory domain (e.g., CD28, 4-1BB, ICOS, or OX40) into the intracellular region. Third-generation CARs further optimize signaling by combining two co-stimulatory domains to synergize activation pathways. Fourth-generation CARs, also known as TRUCKs, build upon second-generation designs by introducing additional genetic elements. Fifth-generation CARs include a truncated cytoplasmic domain of the IL-2 receptor and a STAT3-binding motif, enabling antigen-dependent activation of the JAK-STAT signaling cascade to promote enhanced T cell proliferation and persistence. Created by biorender.

2.1.2 Challenges of CAR-T cell therapy in solid tumors

CAR-T cell therapy has revolutionized the treatment of certain hematologic malignancies. Unlike hematological cancers, solid tumors present a unique and complex set of barriers that limit the effectiveness and safety of CAR-T cell therapy^{119,120}.

One of the first challenges is physical: CAR-T cells must successfully traffic to the tumor site and infiltrate the dense tumor architecture. When CAR-T cells do manage to reach the tumor, they encounter a profoundly immunosuppressive microenvironment. This hostile setting includes regulatory T cells, tumor-associated macrophages, and myeloid-derived suppressor cells, all of which actively inhibit T cell activation. In addition, the TME is often characterized by hypoxia, nutrient deprivation, and the accumulation of immunosuppressive cytokines such as IL-10, TGF-β, and PD-L1. These

factors, together with enzymes like arginase-1, IDO, and inducible nitric oxide synthase, create metabolic and signaling conditions that blunt CAR-T cell function and reduce their persistence^{121–123}. Even if CAR-T cells remain functional within this challenging environment, they face another obstacle: T cell exhaustion. Prolonged antigen stimulation can trigger an intrinsic exhaustion program in T cells, rendering them less effective or completely dysfunctional over time¹²⁴.

Moreover, tumors can adapt by downregulating or completely losing the target antigen, a phenomenon known as antigen escape, which allows them to evade immune detection altogether¹²⁵. This is particularly problematic in solid tumors, where antigen expression is often heterogeneous, varying in both intensity and spatial distribution across tumor cells. To overcome this, strategies such as targeting multiple antigens, combining CAR-T cells with checkpoint inhibitors, or engineering T cells to promote epitope spreading are actively explored. However, these innovations are still under investigation and not yet widely adopted in clinical settings.

Another significant challenge is the potential for serious toxicities. Upon infusion, CAR-T cells can trigger an intense inflammatory response known as cytokine release syndrome (CRS). This condition is characterized by high levels of inflammatory markers (such as IL-6, TNF- α , ferritin, and C-reactive protein) and clinical symptoms including fever, hypotension, and myalgia. In some cases, CRS is followed by neurotoxicity, often referred to as immune effector cell-associated neurotoxicity syndrome (ICANS), which can cause confusion, seizures, or even cerebral edema. Although treatments like tocilizumab (an anti-IL-6 receptor antibody) are effective at managing CRS, they do not consistently prevent or reverse neurological symptoms^{126,127}.

Additionally, CAR-T therapy carries the risk of on-target off-tumor toxicity. This occurs when normal tissues that express low levels of the targeted antigen are mistakenly attacked. A striking example was seen in an early clinical trial involving a high-affinity third generation CAR-T cells against HER2, where low HER2 expression in lung tissue led to a fatal inflammatory reaction shortly after cell infusion¹²⁸.

Given these challenges, current CAR designs must continue to evolve. Ultimately, the success of CAR-T therapy in solid tumors will depend on our ability to develop smarter, safer, and more adaptable therapeutics capable of overcoming the complex barriers inherent to CAR-T cell therapy.

2.1.3 Clinical experience of CAR-T cell therapy in solid tumors

Interim results from a recent phase II trial with Claudin18.2-targeted CAR-T cells (Satri-cel) have shown a significant improvement in progression-free survival (3.25 months in the satri-cel group and 1.77 months in the physician's choice (TPC) group; one-sided log-rank $p < 0.0001$), in heavily pre-treated patients with CLDN18.2-positive gastrointestinal cancers. The ORR was 22% in the satri-cel group versus 4% in the TPC group. CRS G3 occurred in 5% of patients in the satri-cel group¹²⁹. Claudin 6 (CLDN6) might be another interesting target, and a phase 1/2 clinical trial (BNT211-01) is currently underway to evaluate a combination of CLDN6-targeted CAR-T therapy and an RNA-based amplifying vaccine in patients with relapsed or refractory CLDN6-positive solid tumors. Preliminary results from 21 evaluable patients have demonstrated an ORR of 33% and a disease-control rate (DCR) of 67% including one complete response. The treatment has shown a manageable safety profile¹³⁰.

Interestingly, CAR-T cells targeting prostate-specific membrane antigen, transduced with a negative transforming growth factor (TGF)- β receptor, have been evaluated in patients with castration-resistant prostate cancer (CRPC), with modest results (stable disease in 5/13 patients) and a globally good safety profile¹³¹.

GD2-targeted CAR-T cell therapy has shown promising results in pediatric patients with relapsed or refractory high-risk neuroblastoma (ORR 63%, including 9 complete responses among 27 patients)¹³². Another study showed the feasibility and preliminary signs of efficacy of regionally delivered GD2-CAR-T cells in patients with H3K27M-mutated pediatric diffuse midline glioma¹³³. In line with these encouraging results, the interleukin 13 receptor (IL-13R) has also been identified as a promising target in several solid tumors²⁵; the intrathecal administration of IL-13R α 2-targeting CAR-T cells has shown promising efficacy in patients with recurrent high-grade glioma²⁶, and there is

an ongoing phase I clinical trial in patients with advanced melanoma (NCT03119024). Another study involving CARv3-TEAM-E cells (CAR T cells engineered to target both the tumor-specific epidermal growth factor receptor variant III (EGFRvIII) and wild-type EGFR through the secretion of a T cell-engaging antibody molecule (TEAM)) demonstrated rapid and substantial tumor regression within days of treatment in patients with glioblastoma¹³⁴.

In malignant pleural mesothelioma, a phase I trial reported positive data for the intrapleural administration of mesothelin-targeted CAR-T therapy in combination with pembrolizumab, based on preclinical data suggesting an enhancement of CAR-T cells function by PD-1 blockade²⁷. Tyrosinase related protein 1 (TYRP1) has recently been identified as a potential target for CAR-T cell therapy against cutaneous and non-cutaneous melanoma, with interesting preliminary efficacy in patient-derived murine models²⁸.

Collectively, these clinical trials highlight the promising potential of CAR-T cell therapy in the treatment of solid tumors. Studies in patients with solid tumors show that CAR-T cells can effectively infiltrate the TME and exhibit antigen-specific activity. Although some clinical responses have been observed, the majority of patients either fail to respond, relapse early, or experience treatment-related toxicities. In the context of solid tumors, the main adverse events have been on-target off-tumor toxicities that occur when healthy tissues expressing low levels of the target antigen are damaged¹³⁷. The most obvious strategy to avoid toxicities is to target antigens that are exclusively expressed on tumor cells, but not in normal tissue. However, finding suitable antigens to target solid tumors has proven challenging because of their shared expression in healthy tissues and the risk of on-target off-tumor toxicity. An alternative to targeting shared antigens between tumor and normal cells is to tune the affinity of the antigen recognition domain, so that it can differentiate between tumors and normal tissues based on different levels of antigen expression¹³⁸.

In one of the first clinical trials reported, treatment with high dose of high-affinity CAR-T cells targeting HER2 (10^{10} CAR-T cells) caused lethal toxicity in a patient with

colorectal cancer due to T-cell accumulation in the lungs¹²⁸. However, in more recent studies, CAR-T cells targeting HER2 using a different scFv proved to be safe and showed promising antitumor activity in some patients with sarcoma, including a complete response in a pediatric patient^{139,140}.

To date, no studies have been published for the use of HER2-targeted CAR-T cells in BC treatment. However, there are eleven ongoing Phase 1 clinical trials (<https://clinicaltrials.gov/>), all based in the United States and China. Five of them are evaluating HER2-targeted CAR-T cells as monotherapy for the treatment of solid tumors (NCT06101082, NCT05745454, NCT04511871, NCT06241456) or sarcoma (NCT00902044). The remaining studies involve dual CAR-T cell approaches for advanced sarcoma (NCT07066982) and solid tumors (NCT04684459); bispecific CAR-T cells for mBC (NCT06251544); intraventricular delivery in patients with HER2-positive brain metastases (NCT03696030); combination strategies with oncolytic viruses (NCT03740256) or antibody-based biologic (NCT06878248) in solid tumors.

Given that CAR-T therapy has a different mechanism of action compared to trastuzumab, pertuzumab, T-DM1, and T-DXd—where target cell destruction can occur through various apoptosis-inducing pathways such as osmotic lysis (mediated by perforin and/or granzyme B) or FAS-FAS ligand interaction^{141,142}—the development of a CAR-T therapy targeting HER2 would represent a major breakthrough, particularly in the context of solid tumors.

Despite the limited number of HER2-targeted CAR-T studies in other solid tumors, existing data from these models are encouraging and support further exploration. In line with this, and prior to the present work, our group initially developed a putative clinical candidate CAR targeting HER2. This construct was based on a previously described CAR incorporating a low-affinity variant of the 4D5 scFv derived from trastuzumab whose use in CAR-T cells was previously associated with severe toxicity in a clinical case¹²⁸. The humanized 4D5.5 scFv differs from the humanized 4D5 scFv by two amino acid substitutions: one in the light chain (a tyrosine to glutamic acid change at position 55 in CDR2), and another in the heavy chain (a tyrosine to valine change at

position 102 in CDR3). This lower affinity version has been shown to improve safety without compromising antitumor efficacy¹⁴³.

Based on a series of in vitro and in vivo preclinical studies, and following a comparative evaluation of multiple transmembrane and co-stimulatory domain combinations, a CAR construct incorporating the 4D5.5 single-chain variable fragment (scFv), CD8 α as the transmembrane domain, and 4-1BB as the intracellular co-stimulatory domain was selected. This configuration, hereafter referred to as HER2-CD8 α -BBz, demonstrated superior antitumor efficacy, despite exhibiting limited expansion in vitro, presumably due to tonic signaling.

2.1.4 Tonic signaling in 4-1BB-based CAR-T cells

Tonic signaling, defined as ligand-independent activation of CAR-T cells, poses a significant challenge for maintaining T cell functionality and persistence. High levels of CAR expression on the T cell surface have been shown to promote spontaneous receptor clustering, leading to ligand-independent activation of downstream signaling pathways^{144,145}. In the context of 4-1BB-containing CARs, this can induce constitutive activation of the TNF receptor-associated factor 2 (TRAF2) pathway. Sustained TRAF2 activity results in phosphorylation of the I κ B kinase (IKK) complex, leading to NF- κ B activation and transcriptional upregulation of pro-apoptotic molecules such as FAS (CD95) and FAS ligand (CD95L), which have been shown to co-localize at the cell membrane. This cascade ultimately culminates in caspase-8–dependent activation-induced cell death (AICD), compromising T cell expansion and viability¹⁴⁶ (**Figure 9**).

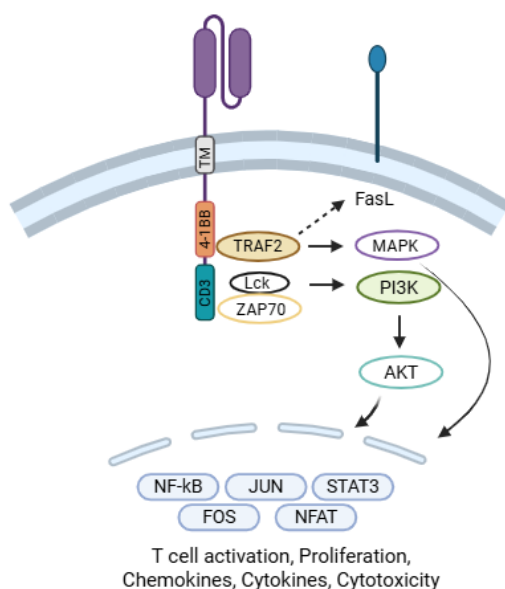


Figure 9. Pathways activation induced by 4-1BB CARs. CARs containing the 4-1BB co-stimulatory domain can induce constitutive activation of the TRAF2 pathway, leading to NF- κ B activation and upregulation of FAS/FASL. At the molecular level, tonic signaling may also activate the PI3K pathway through LCK autophosphorylation. Created with biorender.

Gomes-Silva et al.¹⁴⁶ provided key evidence of this mechanism, demonstrating that CAR-T cells engineered with 4-1BB alone proliferated 70% more slowly during ex vivo expansion, displayed a 4-fold increase in apoptosis, and progressively downregulated CAR expression. Further analysis revealed constitutive phosphorylation of CD3ζ immunoreceptor tyrosine-based activation motifs (ITAMs) and tonic 4-1BB signaling via TRAF2, indicating sustained NF-κB pathway activation in the absence of antigen stimulation. While 4-1BB costimulation is generally associated with beneficial effects—such as improved mitochondrial fitness, greater persistence, and enhanced memory phenotype—its dysregulated activation through tonic signaling can have the opposite effect, promoting apoptotic signaling. This paradox underscores the importance of regulation of 4-1BB signaling in CAR design.

At the molecular level, the signaling cascade activated by CAR engagement parallels that of the endogenous TCR. It involves autophosphorylation of Lck, phosphorylation of CD3ζ and recruitment of ZAP70, followed by activation of downstream effectors such as NFAT and NF-κB¹⁴⁷. However, even in the absence of antigen, low-level Lck activity in naïve T cells can maintain basal phosphorylation of CD3ζ ITAMs, potentially contributing to background tonic signaling—especially in CAR constructs with high surface density. In addition to the NF-κB pathway, tonic CAR signaling can influence other key regulatory circuits such as the PI3K/AKT/mTOR axis^{148,149}, which governs T cell metabolism, survival, and effector function. Persistent, unregulated activation of this axis may skew T cell differentiation or induce metabolic exhaustion, further exacerbating dysfunction.

Understanding the mechanisms underlying tonic signaling in 4-1BB-based CARs is critical for optimizing CAR-T cell therapies and enhancing their safety, persistence, and antitumor efficacy in clinical settings.

HYPOTHESIS AND OBJECTIVES

Central hypothesis

The tumor immune microenvironment plays a fundamental role in determining prognosis and response to HER2-targeted therapies in HER2-positive BC. A detailed understanding of these immune processes, particularly those reflected by the IGG signature, can provide insights into tumor–immune interactions and guide the development of novel immunotherapeutic approaches. Building on this knowledge, HER2-targeted CAR-T cell therapy represents a promising strategy to overcome resistance to current HER2-targeted agents and improve outcomes in patients with advanced disease.

Overall objectives

The overarching aim of this thesis is to integrate a comprehensive characterization of the TME with the development of an innovative HER2-targeted CAR-T cell therapy, creating a translational framework that spans from immune biology to therapeutic innovation.

1. To characterize the TME of HER2-positive BC, with a particular focus on the biological and clinical implications of the IGG signature. This will include studying TILs, TLS, immune receptor clonality, and single-cell transcriptomics to better understand how immune infiltrates influence therapeutic response and survival outcomes.
2. To investigate strategies for the development and optimization of HER2-targeted CAR-T cell therapy, with emphasis on addressing challenges such as tonic signaling, evaluating preclinical evidence on efficacy and safety, and considering requirements for future clinical translation in resistant HER2-positive mBC.

Translational Continuum

Together, these two studies represent complementary aspects of a unified research program. Study 1 (IGG Project) focuses on unraveling the biological significance of the immune microenvironment in HER2-positive BC, while Study 2 (ARI-HER2) applies this

knowledge to the development of a next-generation CAR-T cell therapy. This bench-to-bedside approach underscores the ultimate goal of the thesis: to improve the understanding and treatment of HER2-positive BC through immune-centered strategies.

STUDY 1: IGG PROJECT

HER2DX is the first integrated prognostic assay combining clinicopathological and genomic variables for early-stage HER2-positive BC. HER2DX generates two independent scores: one that predicts long-term prognosis (i.e., risk score) and another that estimates the likelihood of achieving a pCR to neoadjuvant therapy (i.e., pCR score). HER2DX identifies patients with favorable outcomes who may not require therapies beyond chemotherapy and trastuzumab, as well as high-risk patients who may benefit from additional anti-HER2 agents. Thus, HER2DX serves as a valuable tool to guide both de-escalation and escalation strategies in early-stage HER2-positive BC.

HER2DX scores are generated from four distinct gene expression signatures encompassing 27 genes that capture key biological processes: immune activation, luminal differentiation, tumor cell proliferation, and HER2 amplicon expression. Within these, immune activation is represented by an IGG signature, which is uniquely associated with both improved response to neoadjuvant anti-HER2 therapy and superior survival outcomes. Nevertheless, the biological mechanisms underlying this signature remain poorly understood. Elucidating the role of the IGG signature in shaping antitumor immunity is therefore essential, not only to refine prognostic tools but also to inform the development of novel immune-based therapies, such as HER2-directed CAR-T cells.

Hypothesis

HER2-positive BCs with elevated IGG signature expression are enriched in immune infiltrates with clonal diversity that modulate therapeutic response.

Objectives

1. Characterize the microenvironment of tumors with different IGG signature levels and evaluate associations with clinicopathological features, including TILs, TLS, and patient outcomes (published).

2. Explore the IGG signature at the cellular level using single-cell RNA sequencing (published).
3. Assess TCR/BCR clonality in HER2-positive tumors stratified by IGG signature levels (published).

STUDY 2: ARI-HER2 PROJECT

HER2-targeted therapies, including trastuzumab, pertuzumab, T-DM1, T-DXd, and tucatinib, have dramatically improved the prognosis of patients with HER2-positive BC. Despite these advances, a significant proportion of patients eventually develop resistance. Reported mechanisms include alterations in HER2, impaired internalization and lysosomal degradation of HER2–drug complexes, and chemotherapy resistance linked to antibody–drug conjugates. Patients progressing under these circumstances face limited treatment options, underscoring the urgent need for novel therapeutic strategies.

Hypothesis

CAR-T cell therapy offers a distinct mechanism of action, independent of HER2 internalization or lysosomal processing. Once activated, CAR T-cells induce apoptosis of target cells through perforin/granzyme-mediated lysis and FAS–FASL interactions. We hypothesize that HER2-directed CAR-T cells represent a promising alternative for patients with resistance to current HER2-targeted agents.

In a previous project led by Dr. Sonia Guedan's Lab, a HER2-targeted CAR candidate incorporating a 4-1BB co-stimulatory domain demonstrated robust efficacy in preclinical models but showed limited expansion in vitro due to tonic signaling. We hypothesize that attenuating basal signaling will improve T-cell proliferation and persistence, facilitating clinical translation.

Objectives

1. Evaluate and optimize culture conditions that reduce tonic signaling in 4-1BB–based HER2 CAR-T cells.
2. Assess the antitumor efficacy of optimized HER2-targeted CAR-T strategies in preclinical models.
3. Characterize the safety and toxicity profile of the lead CAR-T candidate.

4. Design a clinical trial for HER2-positive mBC and prepare regulatory submissions.

MATERIAL AND METHODS

STUDY 1: IGG PROJECT

1. Clinical samples

To study the immune component of BCs with different levels of IGG signature, 84 formalin fixed-paraffin embedded (FFPE) samples from patients with HER2-positive early-stage BC treated at Hospital Clinic of Barcelona ($n = 74$) and Institut Català d'Oncologia ($n = 10$) were selected. The proportion of stromal TILs was determined on hematoxylin–eosin (H&E) slides according to the International TILs Working Group Guidelines^{150,151}. The presence of TLSs was defined as spatially organized, non-encapsulated areas of immune cell aggregates on H&E slides. Additionally, six biopsies from patients with untreated HER2-positive BC were obtained to conduct single-cell RNA-seq.

2. Gene expression analysis of clinical samples

RNA was extracted from FFPE tumor diagnostic samples using the High Pure FFPE RNA Isolation Kit (Roche, Indianapolis, IN). One to five 10- μ m FFPE slides depending on tumor cellularity were used for each tumor sample, and macrodissection was carried out, when needed, to avoid normal tissue contamination. A minimum of 100 ng of total RNA was analyzed on the nCounter platform (NanoString Technologies, Seattle, WA) using a 192-gene custom panel, including 14 genes of the IGG signature, which was determined using R software v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria).

3. GeoMx DSP data acquisition and analysis

FFPE tumor biopsies from 85 patients were stained with fluorescently labeled CD45 and PanCK antibodies, DAPI (4',6-diamidino-2-phenylindole), and a multiplexed panel of protein antibodies that contained a photocleavable indexing oligonucleotide, enabling subsequent readouts. Regions of interest were selected on the GeoMx DSP platform (NanoString Technologies¹⁵²), segmented according to CD45 and PanCK staining and illuminated using UV light. Released indexing oligonucleotides from each area of interest (AOI) were collected and deposited into designated wells on a microtiter plate, allowing for well indexing of each AOI during nCounter (NanoString

Technologies) readout. For each tissue sample, counts for each marker were obtained from an average of 4.8 (range 1-12) CD45-positive (CD45+) AOIs. Raw protein counts for each marker in each AOI were generated using nCounter. The raw counts were normalized with the AOI surface area. All normalized counts were transformed in log2. Median centering was performed to normalize data across experiments and reduce inter-experimental variation.

4. TCR and BCR sequencing

DNA from FFPE tumor samples with different IGG signature levels was purified with the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions and BCR and TCR were sequenced from 23 and 33 DNA samples, respectively, using the immunoSEQ Assay (Adaptive Biotechnologies, Seattle, WA). The somatically rearranged Homo sapiens TCR locus 3 (CDR3) and BCR immunoglobulin heavy chain (IgH) locus CDR3 were amplified from DNA samples using a two-step, amplification bias-controlled multiplex PCR approach. CDR3 and reference gene libraries were sequenced on an Illumina (San Diego, CA) instrument according to the manufacturer's instructions. Raw sequence reads were demultiplexed according to Adaptive's proprietary barcode sequences. Demultiplexed reads were further processed to remove adapter and primer sequences, and identify and remove primer dimer, germline, and other contaminant sequences. The filtered data were clustered using both the relative frequency ratio between similar clones and a modified nearest-neighbor algorithm, to merge closely related sequences to correct for technical errors introduced through PCR and sequencing. The resulting sequences allowed annotation of the V, D, and J genes and the N1 and N2 regions constituting each unique CDR3 and the translation of the encoded CDR3 amino acid sequence. Gene definitions were based on annotation in accordance with the IMGT database (www.imgt.org). The set of observed biological TCR and BCR IgH CDR3 sequences was normalized to correct for residual multiplex PCR amplification bias and quantified against a set of synthetic CDR3 sequence analogues. Data were analyzed using the immunoSEQ Analyzer toolset.

To characterize the diversity and clonal architecture of the TCR and BCR repertoires, several metrics were calculated¹⁵³:

- **Number of clones (N clones):** The total number of unique TCR or BCR clonotypes identified within the sample, defined by distinct CDR3 sequences and V(D)J gene usage.
- **Productive rearrangements:** Receptor sequences that result in in-frame transcripts without stop codons, leading to potentially functional TCR or BCR proteins.
- **Gini index (Gini):** A measure of inequality in clonal distribution, where 0 indicates perfect equality (all clones equally represented) and 1 indicates maximal inequality (a single dominant clone).
- **Gini-Simpson index (Gini-Simpson):** An index reflecting diversity, calculated as 1 minus Simpson's index ($1 - D$). It represents the probability that two randomly selected sequences belong to different clones. Higher values indicate greater diversity.
- **Simpson clonality:** A metric derived from Simpson's index that emphasizes clonal expansion. Values close to 1 indicate strong dominance of a few clones, whereas values near 0 indicate a more even clonal distribution.
- **Shannon entropy:** A diversity metric quantifying both richness and evenness of the repertoire, calculated from the relative frequencies of all clonotypes. Higher entropy reflects greater diversity.
- **Evenness:** Calculated as a normalized Shannon entropy score, this metric quantifies the distribution of clonotypes within the TCR repertoire. Low evenness values indicate a highly imbalanced repertoire dominated by a few clonotypes, whereas higher values reflect a more uniform distribution across clonotypes.

5. Single-cell RNA sequencing

Fresh core biopsies obtained from six patients with HER2-positive early BC were collected by ultrasound-guided breast biopsy and the tissue (3-5 mm³ approximately) was supplied in 5 ml of RPMI medium. Tumor fragments were dissociated using enzymatic digestion (Human Tumor Dissociation Kit, Miltenyi, Bergisch Gladbach, Germany). Then, CD45⁺ cells were isolated using Human CD45 TIL MicroBeads (Miltenyi) and stored in phosphate-buffered saline containing 0.005% bovine serum albumin. CD45⁺ cells were processed with the 10 Genomics Chromium Controller (10X Genomics Inc.) and single-cell gene expression and TCR/BCR libraries were produced with the Chromium Single Cell 5' Library assay (10X Genomics Inc., Newark, CA), sequenced on an Illumina NovaSeq6000 (100 cycle kit). Sequencing reads were aligned with Cell Ranger Single Cell Software (version 6.1.1, 10X Genomics Inc.) and mapped against the human GRCh38 reference genome (GENCODE v32/Ensembl 98). Quality control, cell annotation, and cluster identification were carried out based on rationale¹⁵⁴.

6. Patient datasets

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC)¹⁵⁵ ($n = 1904$) and The Cancer Genome Atlas (TCGA)¹⁵⁶ ($n = 1082$) BC datasets and pan-cancer TCGA datasets [i.e. cervix ($n = 294$), head and neck ($n = 515$), lung ($n = 510$), melanoma ($n = 443$), and sarcoma ($n = 253$)] were sourced from the cBioPortal for Cancer Genomics (<http://cbioportal.org>),¹⁵⁵ while data from the most recent version of The Sweden Cancerome Analysis Network (SCAN-B)^{157,158} ($n = 6652$) dataset were obtained from Mendeley Data (<https://data.mendeley.com/datasets/yzxtxn4nmd>). Except for the competing risk analyses, patients who experienced BC recurrence were systematically excluded from both datasets. The final analysis comprised 1,133 patients from METABRIC, 4,470 patients from SCAN-B, and 936 patients with BC from TCGA, ensuring a cohort that did not encounter recurrence during the follow-up period. The median follow-up in these patients was 96.9 months in SCAN-B, 185.0 months in METABRIC, and 130.0 months in TCGA. Gene expression was obtained from all datasets. RNA sequencing (RNA-seq) and BCR and TCR repertoire metrics from the

CALGB-40601 neoadjuvant HER2-positive study were obtained from a previously published study¹⁵⁹.

For the pan-cancer analysis, TCGA data were downloaded from cBioPortal.

7. In silico gene expression analysis

Z-scores (METABRIC, TCGA) or log2-transformed (SCAN-B) normalized gene expression values were employed for single genes and for deriving the 14-gene IGG signature, depending on the available gene expression data for each dataset. The Prediction Analysis of Microarray 50 (PAM50) risk of recurrence (ROR) was also determined in the METABRIC and TCGA datasets.

8. Core IGG genes

Genes consistently associated with longevity across METABRIC, SCAN-B, and TCGA datasets were identified as core IGG genes (CIGGs). We further investigated whether the combined assessment of pairs of CIGGs enhanced longevity prediction compared with individual genes. Various mathematical operations, including addition, subtraction, multiplication, and ratio, were employed to combine the expressions of two CIGGs.

9. Statistical analysis

Cumulative incidence functions were used to estimate death without recurrence in a competing risk setting using recurrence as a competing event. Fine and Gray competing risk regression was used to obtain a sub-distribution hazard ratio with 95% confidence interval (CI). In the sensitivity analysis of the subset of patients without recurrence, Kaplan–Meier curves were used to estimate survival outcomes and the log-rank test was used for statistical comparisons. Univariate and multivariable Cox regression and logistic regression models were employed, with the goodness of fit of each model assessed through the chi-square statistic obtained from a likelihood ratio test. The differences in variable distribution were explored by chi-square test. Correlations between two variables were evaluated using the Pearson method. Unpaired t-tests and analyses of variance were employed to ascertain the comparative analysis of numerical variables among different groups. A two-sided α of 0.05 was set as the significance

level for all statistical analyses. No data imputation was carried out. All statistical analyses were carried out using the R software.

STUDY 2: ARI-HER2

1. Cell lines

Tumor cell lines

All human cancer cell lines were purchased from American Tissue Culture Collection (Manassas, Virginia, USA). SKOV3 (ovarian cystadenocarcinoma) was cultured in DMEM-10, MDA-MB-468 in DMEM/F12 (Gibco) and HCC1954 (breast ductal carcinoma) in RPMI-1640 (Roswell Park Memorial Institute, Gibco). All media was supplemented with 10% FBS (Merck, Lot #9669) and with 100mg/mL of penicillin-streptomycin (P/S) and all cell lines were grown at 37°C and 5% CO₂. All cells were regularly validated to be Mycoplasma free.

Genetically engineered tumor cell lines

To generate a cellular model with varying HER2 densities, the MDA-MB-468 cell line was transduced with lentiviral vectors encoding a truncated form of HER2, driven by promoters of low, medium, or high strength. This approach yielded cell lines with negative, low, medium, and high levels of HER2 expression.

HER2 expression in these engineered cell lines was compared to other BC cell lines overexpressing HER2 (**Figure 10 A**). The laboratory-generated MDA-MB-468 HER2 negative or low-expressing cells were representative of tumor cell lines expressing HER2 at physiological levels, whereas the medium or high-expressing MDA-MB-468 cells resembled tumor lines classified as HER2-positive (**Figure 10 C**).

Human primary cells

Human primary cells that express low levels of HER2 including keratinocytes, smooth muscle cells, osteoblasts, endothelial cells, renal cells, cardiomyocytes, langerhans cells, ovarian cells, epithelial pancreatic cells and hepatocytes used for the off-target off-tumor studies were purchased from PromoCell, Accegen and BeCytes Biotechnologies and cultured according to manufacturer's protocols (**Figure 10 B**).

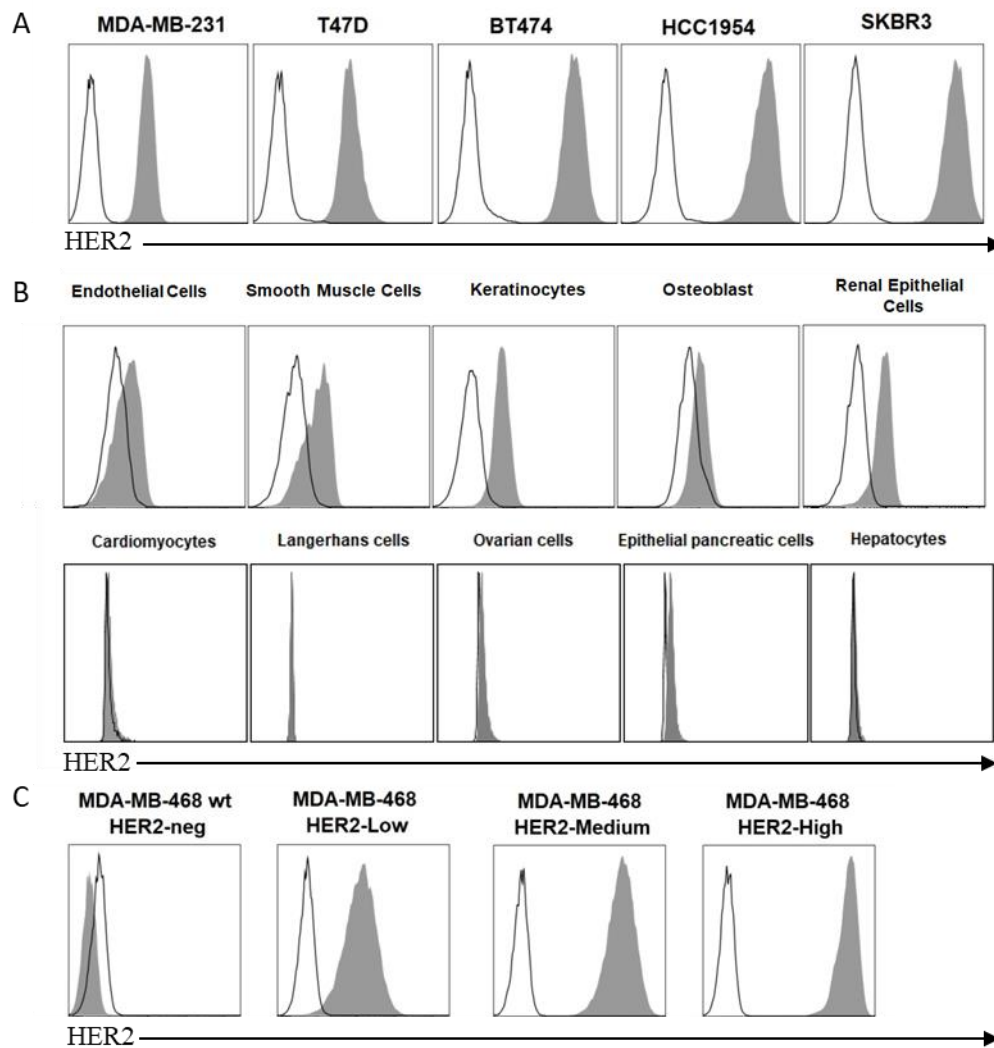


Figure 10. HER2 expression in primary healthy cells and cancer cells. HER2 expression was analyzed by flow cytometry in A) a panel of BC cell lines and B) primary cells. C) The TNBC cell line (HER2 negative) was genetically modified to express low, medium or high levels of HER2 expression. HER2 expression was quantified by flow cytometry as in A and B.

2. Chimeric Antigen Receptor (CAR) development

The HER2-CAR incorporates a humanized 4D5.5 scFv in the distal extracellular position, together with the CD8 α extracellular spacer and transmembrane regions, as well as the 4-1BB co-stimulatory domain and the CD3 ζ signaling domain, in line with second-generation CAR design principles. The 4D5.5 scFv differs from the original 4D5, which was associated with a fatal event in 2010¹²⁸, by two point mutations that reduce binding affinity: one substitution in the light chain (tyrosine to glutamic acid at position 55 of CDR2), and another in the heavy chain (tyrosine to valine at position 102 of CDR3).

In this study, we also used a second-generation CAR with the same domains as the HER2-CAR, but with a different scFv targeting mesothelin (M11).

2.1 Plasmid amplification

Bacteria transformation and culture

Bacteria serve as an effective vector for the amplification of exogenously inserted Deoxyribonucleic acid (DNA), typically in the form of plasmids. This process, known as transformation, enables the replication of the plasmid alongside bacterial DNA, resulting in the production of a large quantity of the vector. Through this mechanism, bacteria efficiently generate amplified copies of the DNA insert. For the transformation of bacteria, the One Shot® Stbl3 chemically competent *E. coli* strain (Thermo Fisher) was used. Bacterial aliquots of 25 µL stored at -80°C were thawed on ice, and a final volume of 2 µL of DNA ligation was added and gently mixed. Subsequently, the mixture was placed on ice for 30 minutes and heat-shocked for 30-45 seconds in a 42°C water bath. Following this, the bacteria were incubated on ice for 2 minutes, immediately resuspended in 200 µL of pre-warmed (37°C) Super Optimal Broth + Catabolic repressor (S.O.C) (Thermo Fisher) and incubated for 1 hour at 225 rpm in a shaking incubator at 37°C. Afterwards, 250 µL of the transformation was plated on pre-warmed LB (Thermo Fisher) agar plates with ampicillin resistance (Sigma-Aldrich) and incubated at 37°C overnight. The next day, isolated colonies of grown bacteria were selected and cultured in 2,5mL of LB media for 8h. The culture was scaled up to 500mL and left to incubate overnight.

Plasmid DNA Isolation

Following bacterial culture, a bacterial pellet is obtained through centrifugation. DNA was isolated using the PureLink™ Quick Plasmid MaxiPrep Kit (Thermo Fisher) that contains the required buffers for bacterial lysis and columns for DNA binding, enabling subsequent DNA purification and elution. The eluted DNA is precipitated with ethanol, washed and resuspended with TE buffer. The concentration of purified DNA is determined with Nanodrop (Thermo Fisher) and adjustment is made to have a stock

with a final concentration of 1000ng/mL. To verify the absence of any mutations throughout the cloned construct, the DNA is sent to Genewiz for sequencing.

2.2 Lentivirus production

Lentivirus (LV) is RNA virus requiring a series of genes (Gag, Pol, Rev, Env, Tat) to be produced. Once LV enters the cell, its RNA genome is retrotranscribed to dsDNA and this can integrate randomly inside the genome.

2.2.1 293FT transfection

Third-generation lentiviral particles were produced by transfecting HEK293FT cells with the transfer vector together with a pre-mixed set of packaging plasmids: pMD2.VSV-G, encoding the envelope glycoprotein VSV-G; pMDLg-pRRE, encoding Gag, which mediates viral core assembly, and Pol, required for reverse transcription; and pRSV-Rev, encoding Rev, essential for post-transcriptional RNA transport.

HEK293FT were seeded at 10×10^6 in a total volume of 18 mL of medium in a p150 culture plate. At the time of transfection, 18 µg of our pCCL transfer plasmid (containing our CAR) and the pre-mixed packaging plasmids mix containing 15 µg of pREV, 15 µg of pRRE and 7 µg of pVSV were diluted in 665µL of serum-free DMEM. In parallel, linear PEI molecular weight (MW) 25K (Polysciences) mixed with medium was added on the DNA mix drop-by-drop and incubated for 20 minutes at RT.

During incubation, 8 mL of medium was removed from each plate, leaving 10 mL per plate. At the end of incubation period, 1.5 mL of the PEI-DNA mixture was carefully added to each p150 plate to avoid disturbing the 293FT cells. After 4-6h of incubation, 18mL of media was replaced, and the plates were returned to the incubator. Viral supernatants were harvested at 48 and 72h post-transfection and centrifuged at 850g for 10 min at 4°C to remove cellular debris. The clarified supernatants were filtered through 0.45 µm filter. Subsequently, one volume of LentiXTM concentrator (Takara Bio) was mixed with three volumes of viral

supernatant by gentle inversion and incubated for 30 minutes at 4°C, followed by centrifugation at 1500g for 45 minutes at 4°C. Finally, the off-white pellet was resuspended in complete medium, aliquoted in 200µL, and stored at –80 °C until use.

2.2.2 Lentiviral titration

Lentiviral titers were determined based on CAR expression in Jurkat cells. Briefly, 2×10^5 Jurkat cells/mL were seeded per well in 96-well flat bottom plates. For each lentiviral preparation, eight wells were used to generate 3-fold serial dilutions. After preparing the dilutions, a fixed volume of each diluted LV was added to the corresponding wells containing Jurkat cells.

Three days post-transduction, cells were harvested and stained to determine the percentage of CAR positivity in the different conditions of diluted LV. The dilution at which fewer than 20% of Jurkat cells were CAR-positive was considered the most accurate limiting-dilution viral titer for that vector preparation. Viral titers were calculated using the following formula:

$$\text{Titer (TU/mL)} = (\% \text{ positive}/100) \times 2^4 \times 20 \times \text{dilution}$$

As transduction efficiency in primary T lymphocytes is approximately one-third of that observed in Jurkat cells, the calculated Jurkat-based titers were adjusted by a correction factor of 3 to estimate the effective viral titer in T cells.

2.3 Isolation, transduction and CAR-T cell expansion

Human T cells were isolated from healthy donor buffy coats obtained from Banc de Sang i Teixits (Barcelona) under the Institutional Review Board approval and informed consent.

Day 0: T cell isolation and stimulation

T cells were isolated using Ficoll gradient (Lymphoprep, Stem Cell Technologies). CD4+ and CD8+ T cells were negatively isolated using RosetteSep™ Human CD4+ or CD8+ T-

Cell Enrichment Cocktails (Stem Cell Technologies) according to manufacturer’s protocol. The buffy coat was mixed with isolation buffer (PBS supplemented with 2% FBS) and the enrichment cocktail, and incubated at room temperature for 25 minutes. After this, isolation buffer was added to reach a final volume of 30 mL, and carefully layered over 15 mL of Lymphoprep. Samples were centrifuged at 1200g for 25 minutes at room temperature with maximum acceleration and deceleration set to 1. The cell layer was collected using a sterile plastic Pasteur pipette, washed with isolation buffer and treated with ACK lysing buffer (Gibco) to remove erythrocytes. Cells were washed again and resuspended in CAR-T culture medium (RPMI 1640 (Gibco) supplemented with 10% FBS, 1x P/S, 10mM HEPES and 1x GlutaMax).

CD4⁺ and CD8⁺ T cells were counted and stimulated separately with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher) at bead-to-cell ratios of 1:1 and 2:1, respectively. CD8⁺ T-cell cultures were supplemented with 10% CD4⁺ T cells to ensure its expansion. To promote growth and proliferation, human IL-7 and IL-15 (Miltenyi) were added every other day at a final concentration of 10 ng/mL until day 8 of expansion. T cells were maintained at a density of 1 × 10⁶ cells/mL in the appropriate plates or flasks, according to the following formula (**Table 1**):

$$N = \text{Total million cells} / 0.35$$

Table 1. Plate or flask to culture T-cells. Appropriate plate/flask to culture T-cells depending on the N value calculated with the indicated formula.

N	Well plate/flask type
2-5	24 well plate
5-8	12 well plate
8-20	6 well plate
20-40	T25 flask-horizontal
40-112.5	T75 flask-horizontal
>112.5	T150 flask-horizontal

Day 1: T cell transduction with lentiviral vectors

Approximately 24h after activation, T cells were transduced with the appropriate volume of CAR-encoding lentiviral vectors at a multiplicity of infection (MOI) of 2-10, in accordance with the formula: MOI = (Viral titer x Virus volume) x T cell number

Day 3: T cell feeding

To prevent T cells from remaining inactive for more than 2 days without fresh medium, half of the culture volume was replenished with CAR-T medium. For conditions involving treatment on day 3 (D3), cells were exposed to the following inhibitors: 1, 5, or 10 μ M AKT inhibitor (Merck); 1, 5, or 10 μ M PI3K inhibitor (Selleck Chemicals); 2 μ g/mL LFA inhibitor (BioXCell); 2 μ g/mL Fas ligand inhibitor (BD Biosciences); or dasatinib (BMS) at concentrations ranging from 6 to 1000 nM.

Day 4: Debeading

On the fourth day of culture, beads were removed in the morning. Concisely, T-cells were gently pipetted up and down to minimize air bubbles, and live T-cells were counted using trypan blue exclusion. CAR-T cell medium for each condition was calculated to adjust the cell concentration to 1×10^6 cells/mL. Samples were placed on a DynaMag-2 or Dynal 15-mL magnet magnet, depending on the volume (Thermo Fisher), allowing the beads to accumulate on the magnet-side walls for one minute. The previously calculated medium was used to wash the beads after each transfer, and the cells were fed with the washing medium. For conditions involving treatment on day 4 (D4), cells were exposed to the following inhibitors: 1, 5, or 10 μ M AKT inhibitor (Merck); 1, 5, or 10 μ M PI3K inhibitor (Selleck Chemicals); 2 μ g/mL LFA inhibitor (BioXCell); 2 μ g/mL Fas ligand inhibitor (BD Biosciences); or dasatinib (BMS) at concentrations ranging from 6 to 1000 nM.

Day 6 to 10: T cell expansion

CAR-T cells were left undisturbed for 24 hours and subsequently counted daily. Cells were maintained at a density of 0.8×10^6 cells/mL to prevent overgrowth and transferred to the appropriate flask (Table 1) until day 10-11, when CD4 and CD8 T cells were mixed at a 1:1 ratio considering that the CD8 groups contain around a 10% of CD4 T cells, and cryopreserved.

To account for differences in transduction efficiency between groups, CAR⁺ expression was normalized by supplementing cultures with untransduced (UTD) T cells when necessary.

Population doublings were calculated from day 4 until the end of primary T cell expansion, relative to the number of cells stimulated on day 0, using the next formula:

$$\text{Population Doubling} = (\log_2 \text{Total cells}_{\text{day N}}) - (\log_2 \text{Total cells}_{\text{day 0}})$$

In experiments requiring continuous exposure to dasatinib, 50 nM of the drug was added daily to the culture.

3. Flow cytometry

CAR surface detection

At least 200,000 T-cells were collected from wells and transferred into either a 96-well plate (U-bottom) or FACS tubes, followed by centrifugation at 2000 rpm for 3 minutes and removal of the SN. Subsequently, cells were resuspended in 200 µl of PBS, and after another round of centrifugation, the supernatant was discarded. A single wash with 200 µl of PBS was performed to remove any residual FBS, critical for subsequent staining with viability dyes. Following this, cells were stained with Live and Dead (L/D) eFluor™ 450 (ThermoFisher) in the dark for 30 minutes RT and washed two times with PBS.

To detect CAR expression, cells were stained using goat anti-human IgG-biotin (Jackson ImmunoResearch). Due to its recognition of fragments from IgGs, the anti-Fc antibody cannot be combined with other surface antibodies (such as anti-CD4, CD8, etc.) as it poses a risk of nonspecific binding. Therefore, the anti-Fc antibody must be added separately and in advance of the other antibodies, in a separate step. To do this, cell pellets were resuspended with 100 µl of FACS buffer containing 2 µl of biotinylated anti-CAR antibodies. Following four washes with FACS buffer to ensure thorough removal of unbound antibodies, cells were resuspended in 100 µl of FACS buffer containing the antibodies against cell surface molecules and streptavidin. Finally, the cell pellet was fixed in 200 µl of PBS containing 2% paraformaldehyde (PFA). Distinct cell populations were determined in a FACS Canto II or Fortessa5L flow cytometers (BD

Biosciences). To ensure a high quality analysis, no fewer than 10.000 to 20.000 events of the population of interest were acquired and subsequently analyzed using FlowJo Software.

Apoptosis staining

For apoptosis analysis, cells were first processed according to the protocol described for CAR surface detection. At the end of this procedure, instead of performing the fixation step, samples were washed once with PBS and subsequently with 1× binding buffer. Cells were then stained with Annexin V (Thermo Fisher) and incubated for 15 minutes at room temperature in the dark. Finally, the cell pellets were resuspended in 1× binding buffer and analyzed by flow cytometry using either a FACS CANTO II or a Fortessa 5L (BD).

FACS sorting

CAR-T cells were sorted based on CAR expression on the eleventh day of T-cell expansion for experiments with or without dasatinib. Briefly, stained cells were sorted at 4°C using a BD FACS Aria II or SORP cell sorter by the personnel from the Cytometry and cell sorting facility at IDIBAPS. Gating strategies were applied based on forward and side scatter properties to exclude debris and doublets. Subpopulations of interest were identified based on fluorescence intensity corresponding to specific markers. Sorted cells were collected into sterile tubes containing a 50:50 mixture of PBS and FBS to enhance cell viability. After sorting, the sorted cells were assessed by RT-PCR.

5. *In vitro* functional assays

Cytokine release determination

For experiments with tumor cells or primary healthy cells, a total of 1×10^5 cancer cells were seeded in 48-well plates. After overnight incubation, T-cells were added at an effector/target ratio of 3:1. Supernatants were collected 24 hours after coculture, and interferon (IFN)- γ and interleukin (IL)-2 levels were measured using the DuoSet ELISA Development Kit (R&D Systems) according to the manufacturer's protocol.

Samples were incubated on a plates coated with a capture antibody. After sample incubation, a detection antibody was added, followed by the addition of the Avidin-HRP solution. After incubation, substrate solution was added, and positive wells developed a blue color, which was then stopped with a sulfuric acid-containing solution, turning the wells yellow. Absorbance was measured using an Infinite 200 PRO Plate Reader (Tecan) at 450 nm and 570 nm for background subtraction. Protein concentrations were calculated by comparison to a standard curve generated from known concentrations of the target protein.

xCELLigence

To assess the cytotoxic activity of CAR-T cells against adherent target cells, the xCELLigence system was used for real-time monitoring of impedance, allowing the quantification of viable cell attachment. Target cells were seeded onto E-16 plates (Agilent) and incubated in the instrument for approximately 20h to allow cells to adherence and establish baseline impedance. After this initial incubation, effector cells were added, and impedance was recorded at 20-min intervals over a period of 100-150h.

Degranulation assay

CD107a expression on the T cell membrane serves as an indicative marker activation and cytotoxic degranulation. For this assay, target cells were seeded in 48-well plates. After overnight incubation, T cells were added at an effector/target ratio of 1:1 and incubated for 2 hours at 37°C. To enable detection of the CD107a, a protein transport inhibitor containing brefeldin A (GolgiPlug; BD Bioscience) was added to each well together with 2 µL of the anti-CD107a antibody. The co-culture was then extended for an additional 4 hours. Following the total 6h incubation, standard T cell staining was performed for flow cytometry analysis.

6. Gene expression analysis

RNA isolation from cells and clinical samples

Total RNA was isolated from cells using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Briefly, cells were pelleted by centrifugation at 350g for 20

minutes and resuspended in 990 μ L of freshly prepared RLT buffer (QIAGEN) supplemented with 10 μ L of undiluted β -mercaptoethanol (Life Technologies). Cell disruption and homogenization were achieved by vortexing, followed by centrifugation at maximum speed for 2.5 minutes at room temperature. The supernatant was carefully transferred to new low-binding tubes. Next, 70% ethanol was added and mixed vigorously. After centrifugation, the samples were loaded onto QIAGEN RNeasy MiniElute columns, spun, and the flow-through was discarded; this step was repeated until all samples had been processed. To avoid genomic DNA contamination, purified RNA was treated with RNase-Free DNase following 15 minutes of incubation at RT. Further washing steps were carried out with RWI and RPE solutions from the RNeasy Mini kit. Finally, elution was done by adding 14 μ L RNase-free H₂O to the column, incubating, and spinning. Purified RNA was quantified and stored at -80°C until further use in downstream applications.

From FFPE tumor samples RNA was extracted using the High Pure FFPE RNA Isolation Kit (Roche, Indianapolis, IN). One to five 10- μ m FFPE slides depending on tumor cellularity were used for each tumor sample, and macrodissection was carried out, when needed, to avoid normal tissue contamination.

Real-time polymerase chain reaction (RT-PCR)

RT-PCR was performed to determine the copy number per genome in the positive and negative fraction of CAR, in presence or absence of dasatinib, on day 11 after T cell activation (n=3). Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacture's protocol. DNA concentration and purity were assessed using a NanoDrop (Thermo Fisher), and samples were diluted to a concentration ranging from 25 to 100 ng/ μ L.

Amplification reactions were carried out with SYBR Green in combination with customized primers at 10 μ M. The following primer sets were employed: GATA (Forward (Fw): 5'-TGGCGCACAACTACATGGAA; Reverse (Rv): 5'-CGAGTCGAGGTGATTGAAGAAGA), WPRE (Fw: 5'-CGCTGCTTTAATGCCTTTGTAT; Rv: 5'-GGGCCACAACTCCTCATAAA) and HER2 (Fw: TGAATGGGTTGCAAGGAT; Rv:

TGGATGTGTCTGCGCTTATAG). For each target, 100 ng of genomic DNA was used per reaction.

Standard curves were prepared for each gene, ranging from 10^8 to 10^2 copies per genome. RT-PCR reactions were performed in 384-well plates on a LightCycler480 instrument (Roche). Cycling conditions consisted of an initial denaturation phase at 95°C for 5 min, followed by 40 cycles of amplification at 95°C for 10 seconds, 58°C for 10 seconds and 72 °C for 5 seconds. Data acquisition and analysis were conducted using the instrument's software to determine the cycle threshold (Ct) values for each sample. Ct values were normalized to the reference gene GATA. WPRE was used to quantify the total number of CAR molecules per sample.

***ERBB2* expression with nCounter platform**

A minimum of 100 ng of total RNA was analyzed on the nCounter platform (NanoString Technologies, Seattle, WA) using a 192-gene custom panel, including *ERBB2*, which was determined using R software v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria).

CAR-T cell characterization with nCounter technology

Gene expression analysis was performed using the human CAR-T cell characterization panel on the nCounter platform (Nanostring Technologies, Seattle, WA). NanoString uses molecular barcodes that bind targets, are fixed on a cartridge, and quantified by nCounter systems for multiplexed digital detection. Analyses were conducted on UTD control T cells as well as CAR-T cells, either untreated or treated with dasatinib, at different time points.

Samples were prepared according to the manufacturer's guidelines for the nCounter human CAR-T Characterization Panel (Nanostring). This comprehensive panel encompasses 780 human genes across eight critical components of CAR-T biology: metabolic fitness, phenotype, TCR diversity, toxicity, activation, persistence, cell types and exhaustion. Briefly, for each sample, 3'mRNA gene expression profiling was generated from 250 ng of RNA. Next, the target RNA alongside the panel standard, a

synthetic DNA oligonucleotide pool corresponding to the target sequences of the probe sets in the panel used for calibration, were hybridized together with the reporter and capture CodeSet at 65°C for 20 hours. Following hybridization, samples were loaded onto the fully automated nCounter Prep Station (NanoString Technologies, Seattle, WA), where they were purified and immobilized onto the internal surface of a sample cartridge for 2.5 hours. Once this step was completed, the sample cartridges were transferred to the nCounter Digital Analyzer (NanoString Technologies, Seattle, WA) for image acquisition and data processing as per the manufacturer's protocol. The expression level of a gene was measured by counting the number of times the specific barcode for that gene was detected. The barcode counts were then tabulated in a comma-separated value (CSV) format. The raw digital count of expression was exported from nSolver v3.0 software for downstream analysis. Gene expression levels were normalized against the housekeeping genes. Data analysis was conducted in R (v4.3.2). Differentially expressed genes between conditions were explored using the limma package (v3.58.1). Pre-ranked Gene Set Enrichment Analysis (GSEA) was conducted over the ranked list of genes based on the log2 Fold Change obtained from the differential expression analysis, per comparison. GSEA was conducted through fgsea function from fgsea R package (v1.28.0) with default parameters (<http://bioconductor.org/packages/fgsea/>). GSEA interrogated Gene Ontology database through gage package (v2.52.0). Plots were done in R.

7. *In vivo*

All mouse experiments were performed in accordance with protocol (P 184-20), approved by the Ethics Committee for Animal Experimentation (CEEA) of the University of Barcelona and Generalitat de Catalunya.

NOD scid gamma (NSG) mice were selected as the animal model due to their high level of immunodeficiency. The NSG colony was purchased from The Jackson Laboratory. Tumor cell lines were expanded to the required numbers, trypsinized, washed, and resuspended in a 1:1 mixture of PBS and Matrigel (Corning) kept on ice. Matrigel, a solubilized extracellular matrix (ECM), was used to enhance tumor engraftment and

growth, particularly in models with low take rates.

Systemic tumors were established in female NSG mice by subcutaneous inoculation of $4\text{--}5 \times 10^6$ SKOV3 cells in a final volume of 100 μL per flank. Mice were randomized according to tumor size the day before treatment to ensure comparable baseline tumor burden across groups. In parallel, CAR-T cells were thawed at 4×10^6 cells/mL. The following day, viable cells were counted using trypan blue exclusion, washed twice with PBS, filtered through a 100- μm strainer to remove aggregates, and resuspended in PBS. Treatment consisted of a single intravenous injection of $2\text{--}5 \times 10^6$ control T cells or CAR-T cells in 100 μL PBS, administered once tumors reached $150\text{--}350 \text{ mm}^3$.

Tumor dimensions were measured weekly with a digital caliper, and volumes were calculated based on length and width measurements. Mice were sacrificed when tumors reached 1500 mm^3 , ensuring that no animal remained with a tumor burden above this threshold for more than five days.

8. Clinical samples

PBMC

In this study, to evaluate the potential clinical applicability, peripheral blood mononuclear cells (PBMCs) were obtained from two patients with HER2-positive BC. T cells were isolated following the protocol of the EasySep™ Human CD3 Positive Selection Kit (Stemcell Technologies).

FFPE samples

Five inflammatory tissue samples from colitis ($n=5$) and hepatitis ($n=5$), along with HER2-positive ($n=132$) and HER2-negative ($n=142$) BC samples from the Hospital Clínic of Barcelona, were analyzed. HER2 IHC and *ERBB2* gene expression analyses were performed (see section Gene Expression Analysis). The IHC staining was analyzed by the Pathology Department of our center.

HER2 blood test

Serum samples from patients with HER2-positive and HER2-negative BC were collected for the quantification of circulating HER2 protein levels. For all patients (n=8), samples were obtained both before and after chemotherapeutic treatment. In four cases of HER2-positive BC, sequential measurements were performed to evaluate the kinetics of plasmatic HER2 levels. HER2 concentrations were determined using a quantitative immunoassay implemented on the Atellica Solution platform, following the manufacturer's instructions.

9. Statistical analysis

All statistical analyses were performed using GraphPad Prism v9 (GraphPad Software Inc.). Data are presented as mean $\pm$ Standard Deviation (SD). For comparisons between two parametric groups, unpaired Student's t-tests were applied. When comparing three or more groups under a single condition, one-way analysis of variance (ANOVA) with Tukey post hoc test was used. For experiments involving multiple groups or conditions, two-way ANOVA with Sidak or Tukey's multiple comparisons correction were performed.

Survival analyses from in vivo experiments were conducted using the Kaplan-Meier method, and statistical differences between groups were assessed with the log-rank test. A p-value < 0.05 was considered statistically significant. Statistical significance is denoted as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

RESULTS

STUDY 1

IGG PROJECT

Comprehensive characterization of the tumor microenvironment (TME) in HER2-positive breast cancer according to IGG signature

To study the immune component of BC with different levels of IGG signature, 84 FFPE samples from patients with HER2-positive early-stage BC treated at Hospital Clinic of Barcelona (n = 74) and Institut Català d'Oncologia (n = 10) were selected. Among these, 37 samples (44%) were classified as IGG-high, showing a higher pCR rate (62.2%) and a greater prevalence of TILs (48.6%) and TLS (64.9%) compared to samples with lower IGG levels (**Table 2**). Across the entire cohort, hormone receptor positivity and the HER2-enriched intrinsic subtype were predominant.

	IGG High (n=37)	IGG Medium (n=23)	IGG low (n=24)	p-value
TILs				
≥30%	18 (48.6%)	1 (4.3%)	0	<0.001
<30%	19 (51.4%)	22 (95.7%)	24 (100%)	
TLS				
Yes	24 (64.9%)	11 (47.8%)	7 (29.2%)	0.02
No	13 (35.1%)	12 (52.2%)	17 (66.7%)	
pCR				
Yes	23 (62.2%)	9 (39.1%)	8 (33.3%)	0.03
No	12 (32.4%)	14 (60.9%)	16 (66.7%)	
Unknown	2 (5.4%)	0	0	
Intrinsic subtype				
HER2-enriched	21 (56.8%)	7 (30.4%)	14 (58.3%)	0.09
Luminal B	5 (13.5%)	6 (26.1%)	3 (12.5%)	
Luminal A	5 (13.5%)	6 (26.1%)	5 (20.8%)	
Basal-like	5 (13.5%)	1 (4.3)	0	
Normal-like	0	3 (13%)	2 (8.3%)	
Unknown	1 (2.7%)	0	0	
Hormone receptor				
Positive	22 (59.5%)	20 (87%)	16 (66.7%)	0.08
Negative	15 (40.5%)	3 (13%)	8 (33.3%)	

Table 2. Characteristics of HER2-positive early-stage BC FFPE samples by IGG signature status.

*TILs: tumor-infiltrating lymphocytes, TLS: tertiary lymphoid structures, pCR: pathological complete response.

1. IGG-high tumors show increased expression of plasma cell and activated T cell proteins, along with reduced expression of immunosuppressive proteins

To gain a more comprehensive understanding of the TME and its association with the IGG signature, we conducted GeoMx Digital Spatial Profiling (DSP) on stromal regions from a cohort of 85 HER2-positive BC. These tumors had previously been stratified into IGG-high, IGG-medium, and IGG-low groups based on predefined cut-off values derived from the HER2DX genomic test (**Figure 11 A**), which uses extracted bulk RNA.

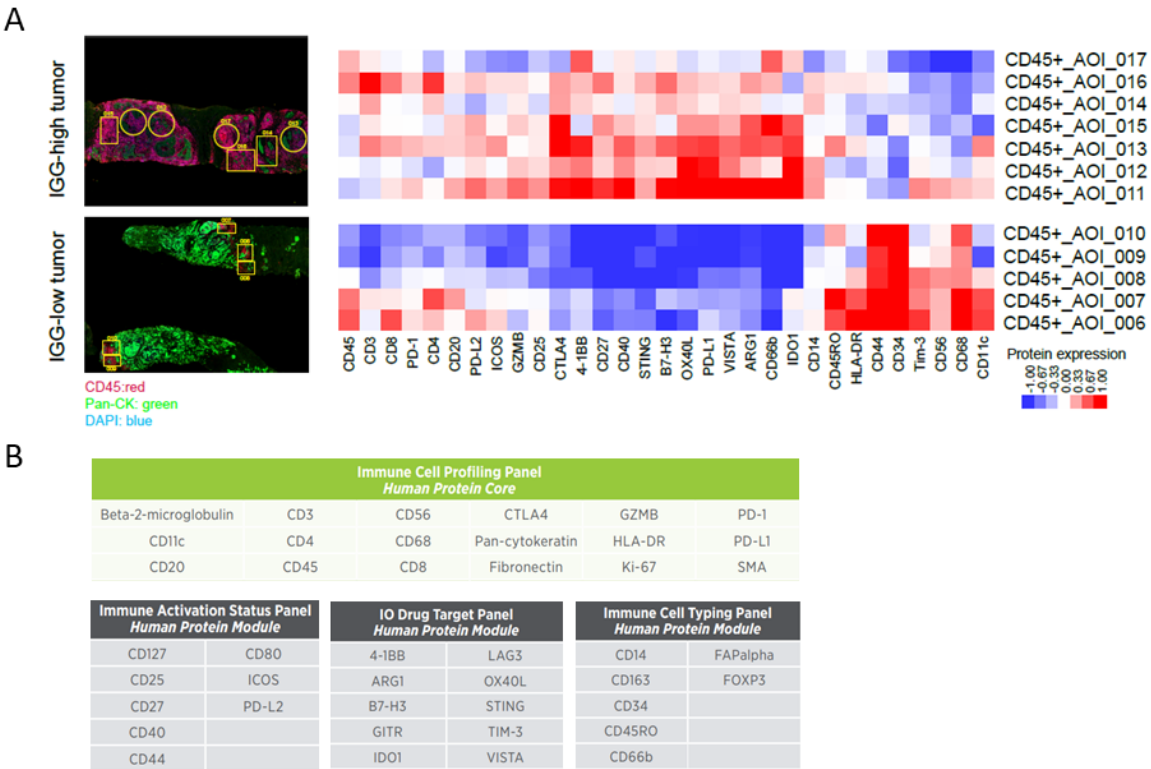


Figure 11. Characterization of the TME in HER2-positive BC. A) Example of protein-based spatial profiling in an IGG-high versus an IGG-low tumor using the GeoMx DSP platform. Tumor cells are shown in green, immune cells in red, and cell nuclei (DNA) in blue. Selected areas of interest (AOIs) are outlined in yellow. The heatmap shows the expression levels of immune-related proteins within CD45+ AOIs for each sample. B) Panel of 42 immune-related proteins analyzed using the GeoMx DSP platform.

For the spatial profiling analysis, in collaboration with a breast pathologist (Dr. Sanfeliu), we selected a total of 407 CD45-positive (CD45+) TME areas of interest (AOIs), corresponding to immune-enriched stromal regions across the cohort. Within these AOIs, we quantified the expression levels of 42 immune-related proteins (**Figure 11 B**), covering a broad spectrum of immune cell types and functional states. Moreover, among the 407 TME AOIs, 87 (21.4%) corresponded to TLS AOIs.

Compared to IGG-medium and IGG-low tumors, IGG-high tumors demonstrated a distinct immune-stromal protein expression profile. Specifically, IGG-high tumors showed significantly elevated levels of markers associated with plasma cells (CD27, $p=0.02$), T cells (CD3, $p=0.03$), and antigen-presenting cells -APCs- (CD40, $p=0.06$ and IDO1, $p<0.001$). In contrast, these tumors exhibited reduced expression of proteins linked to stromal remodeling (fibronectin, $p<0.001$), the memory T cell phenotype (CD45RO, $p=0.007$) and M2-like macrophage infiltration (CD163, $p<0.001$) which are often associated with immunosuppressive TME (**Figure 12**).

These findings suggest that IGG-high tumors are characterized by a more active and immune-inflamed microenvironment, with enhanced APC and T cell recruitment, alongside reduced markers of immune suppression and stromal fibrosis.



Figure 12. IGG-high tumors exhibit an immune-inflamed microenvironment characterized by elevated APC and T cell markers, reduced immune exhaustion and stromal remodeling proteins. Plots display the median-centered expression levels of 42 immune-related proteins across IGG signature groups (high, medium, and low).

2. The IGG signature captures TLS-like immune activity even in TLS-negative tumors

Additionally, we measured the expression of 42 immune-related proteins in 87 TLS-selected AOIs, and compared their profiles to those from non-TLS TME AOIs. TLSs were defined as spatially organized, non-encapsulated aggregates of immune cells within the tumor stroma.

We observed a significant association between IGG signature expression and the presence of TLSs in the TME (**Figures 13 A-B**). Specifically, 56.8% of tumors with TLSs were classified as IGG-high, compared to only 31.7% of tumors without TLSs ($p < 0.001$) (**Figure 13 C**).

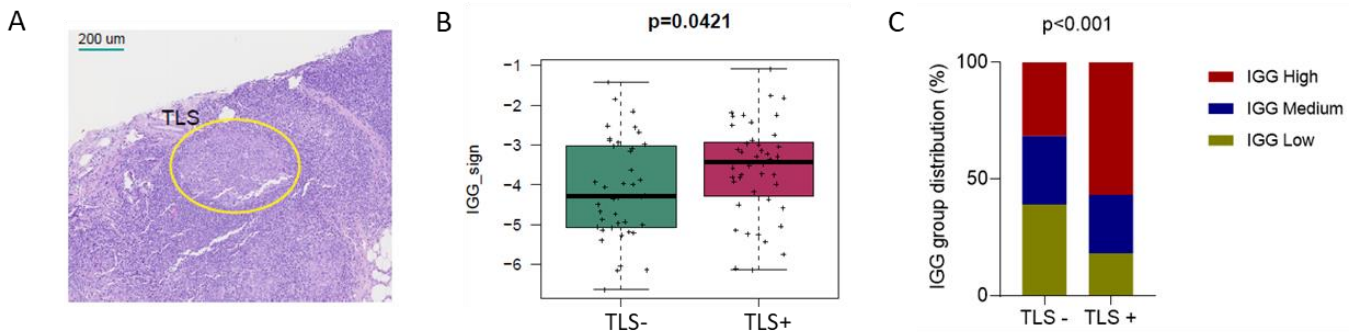
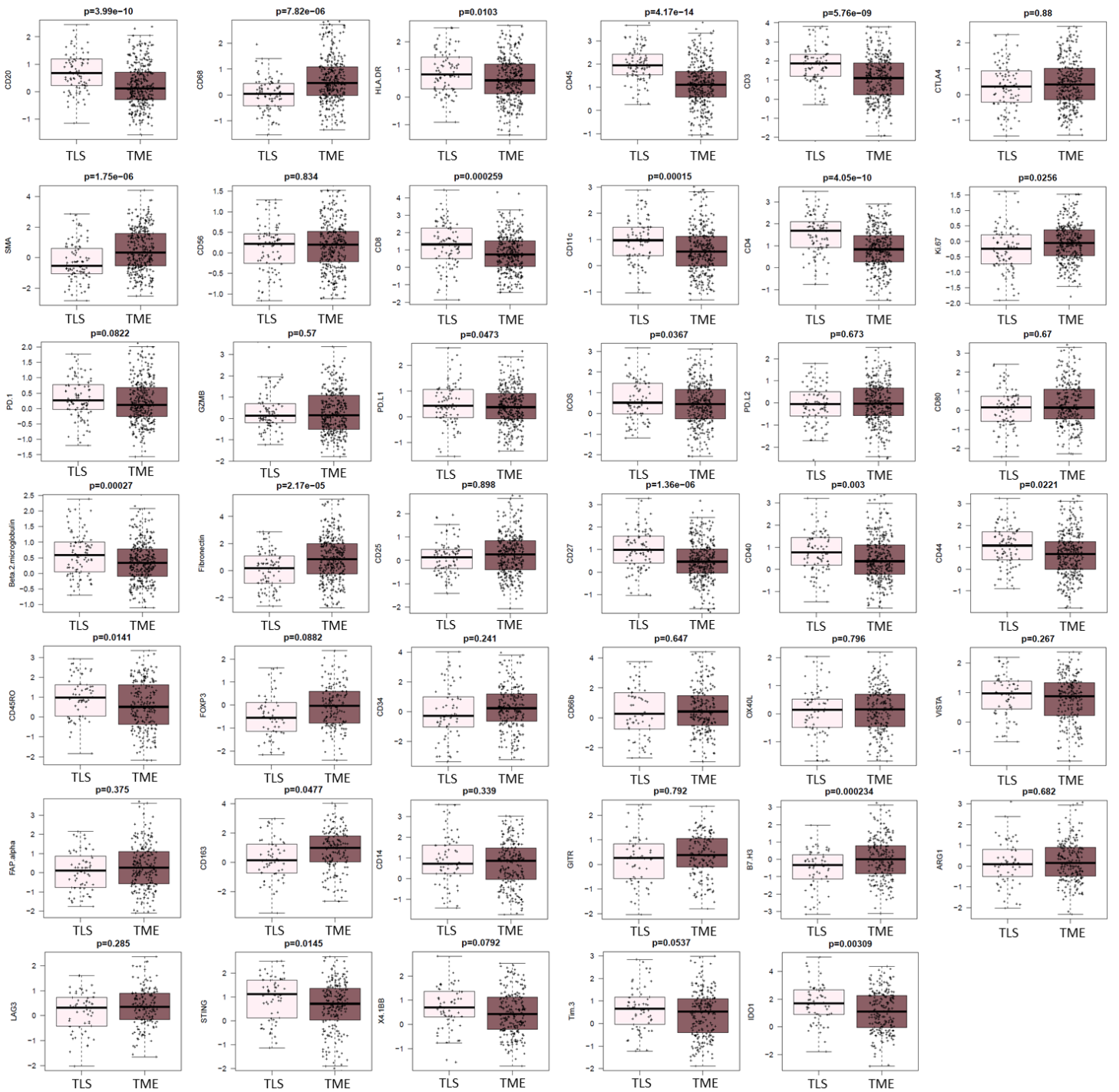


Figure 13. Association between IGG signature expression and the presence of TLSs in the TME. A) Representative H&E staining image showing a TLS in a breast tumor. B) IGG signature expression levels in tumors without TLSs (n=41) versus those with TLSs (n=44). C) Distribution of IGG-high, medium, and low expression groups among TLS-negative and TLS-positive tumors.

To further explore the immune biology associated with TLSs, we focused on immune protein expression patterns within the 87 TLS regions using spatial profiling. A total of 22 out of 42 proteins (52.4%) were found to be differentially expressed between TLS and non-TLS TME regions (false discovery rate < 5%). Notably, immune-related markers such as CD20 (B cells), CD27 (plasma cells), CD11c (dendritic cells), and CD3 (T cells) were significantly upregulated in TLS regions compared to non-TLS areas (**Figure 14**). Conversely, markers associated with stromal remodeling and immune suppression, including SMA, fibronectin, and CD163 (M2-like macrophages), were significantly downregulated in TLS regions (**Figure 14**).



Figure

14. The immune biology associated with TLSs. Plots show the median-centered expression levels of 42 immune-related proteins, stratified by TLS or non-TLS TME regions.

Based on these differentially expressed proteins, we developed a TLS-associated protein signature (**Table 3**). This signature could be detected not only in AOs from non-TLS regions within tumors that contained TLSs but also in tumors without histologically visible TLSs, although statistically significant differences were observed between the

groups (**Figure 15 A**). Moreover, when we applied this signature to tumor samples without histologically detectable TLSs, we observed significantly higher expression levels in IGG-high/medium tumors compared to IGG-low tumors (**Figure 15 B**). These findings underscore the ability of the IGG signature to reflect TLS-like immune activity within the tumor stroma, even in the absence of microscopically identifiable TLSs.

Protein ID	SAM d-score	Fold Change	FDR
CD45	4.38	1.84	0
CD3	3.58	1.73	0
CD4	3.50	1.60	0
CD20	3.46	1.59	0
CD27	2.83	1.51	0
CD8	2.21	1.40	0
CD11c	2.10	1.33	0
CD40	1.84	1.34	0
Beta-2-microglobulin	1.82	1.25	0
HLA DR	1.39	1.20	0
CD44	1.37	1.23	0
ICOS	1.28	1.22	0
PD-L1	1.15	1.18	0
PD-1	0.93	1.13	0
GZMB	0.35	1.06	0
PD-L2	0.26	1.04	0
SMA	-3.14	0.58	0
Fibronectin	-2.94	0.57	0
CD68	-2.52	0.71	0
Ki-67	-1.12	0.87	0
CD56	-0.11	0.99	0
CTLA4	-0.09	0.99	0
CD80	-0.08	0.99	0
CD25	-0.07	0.99	0

Table 3. TLS-associated protein signature identified by unpaired SAM analysis comparing TLS-positive versus TLS-negative samples.*FDR: False discovery rate corresponds to the q-value reported by SAM.

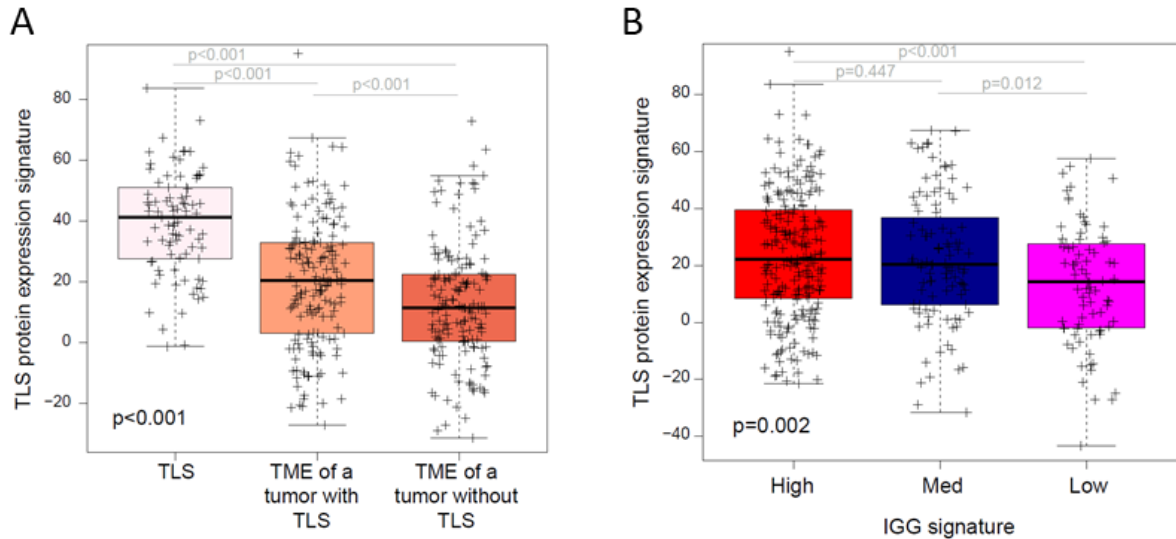


Figure 15. Association of the TLS protein expression signature with TLS presence and IGG. A) Expression of the TLS-associated protein signature in TLS regions, in non-TLS tumor microenvironment (TME) regions of tumors with TLSs, and in TME regions of tumors without TLSs. B) Expression of the TLS-associated protein signature in tumors without histologically detectable TLSs, stratified by IGG signature level (high, medium, low).

Furthermore, IGG expression was significantly associated with TILs, further supporting its role as a marker of an immune-inflamed TME (**Figure 16**).

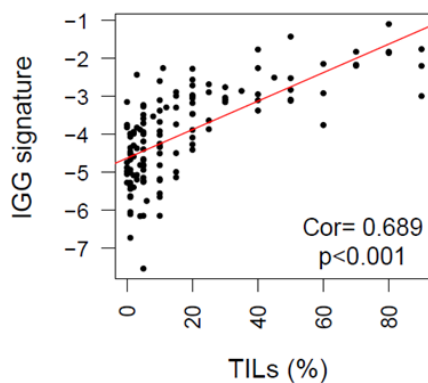


Figure 16. Correlation between IGG signature expression and TILs.

3. Characterization of the IGG signature at single-cell resolution

To dissect the cellular complexity underlying the IGG signature in BC, we performed single-cell RNA sequencing (scRNA-seq) on freshly isolated CD45⁺ immune cells from six independent HER2-positive BC biopsies that exhibited varying levels of IGG signature expression (**Figure 17 A**). In total, six samples were analyzed: three IGG-high, one IGG-medium, and two IGG-low. The IGG-high and IGG-medium samples displayed a similar distribution of immune cell populations. Notably, in the tumor sample with the highest IGG signature score (BC177), 24.0% of CD45⁺ cells were classified as

activated B cells and plasma cells. In contrast, the tumor with the lowest IGG signature score (BC192) showed a markedly reduced presence of these cell types, with activated B cells and plasma cells representing only 1.4% of the total CD45+ population (**Figure 17 B**).

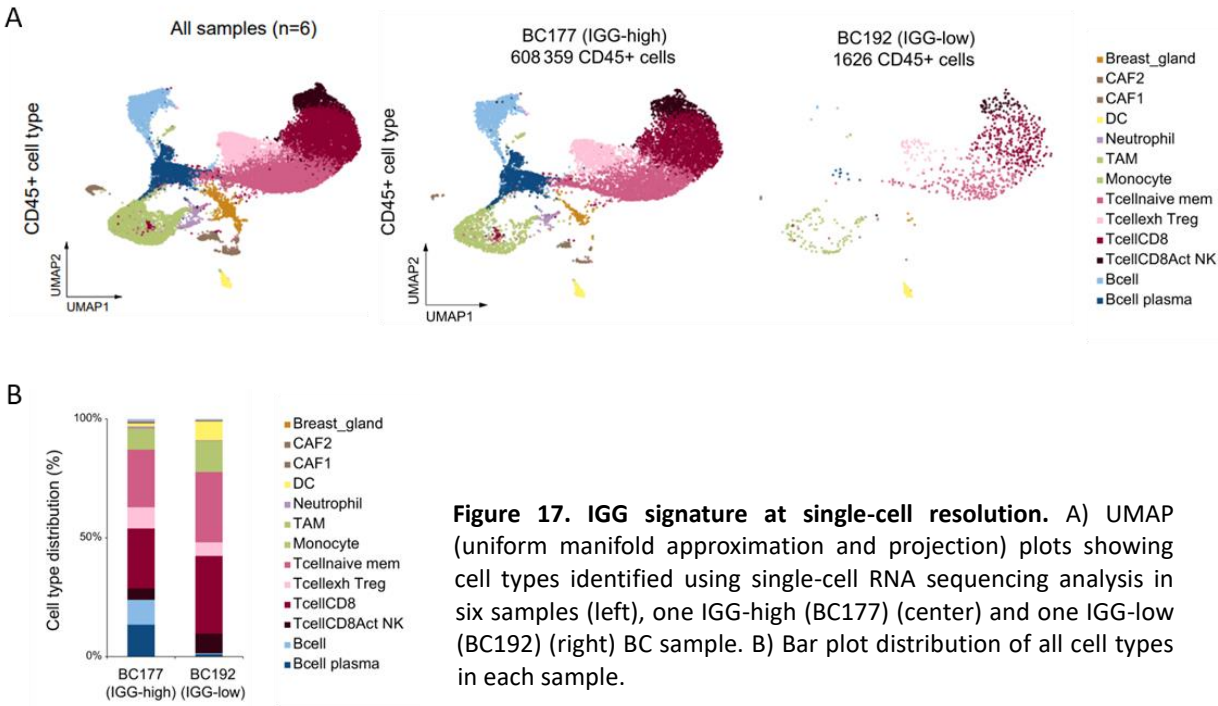


Figure 17. IGG signature at single-cell resolution. A) UMAP (uniform manifold approximation and projection) plots showing cell types identified using single-cell RNA sequencing analysis in six samples (left), one IGG-high (BC177) (center) and one IGG-low (BC192) (right) BC sample. B) Bar plot distribution of all cell types in each sample.

These transcriptomic findings were further validated by IHC staining for key immune cell markers: CD20 (B cells), CD38 (plasma cells), and CD3 (T cells), confirming the differential abundance of these immune subsets across tumors with distinct IGG signature levels (**Figure 18**).

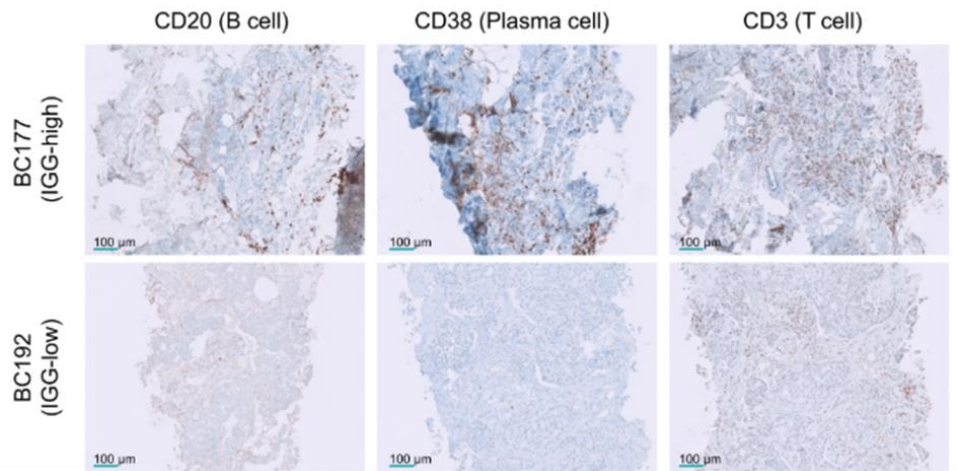


Figure 18. Immunohistochemistry of IGG-high and IGG-low tumors. Representative staining of breast cancer samples BC177 (IGG-high) and BC192 (IGG-low), showing CD20 (B-cell marker), CD38 (plasma cell marker), and CD3 (T-cell marker).

4. Interplay of IGG and B/T-cell clonality

We investigated BCR and TCR clonality in 23 and 33 HER2-positive breast tumor samples, respectively, with previously determined IGG signature expression levels. For this, we used the immunoSEQ DNA-based assay to assess the diversity and clonality of immune receptor repertoires. Overall, tumors with high IGG signature exhibited a greater number of productive rearrangements and increased polyclonality in both B and T cell populations, as indicated by lower Simpson clonality and higher Shannon entropy values—metrics commonly used to quantify repertoire diversity (**Figure 19 A**). To validate these findings, we analyzed an independent external HER2-positive BC dataset (n = 264, CALGB-40601) using in silico methods based on bulk RNA-seq data, which yielded comparable results (**Figure 19 B**). In IGG-high tumors, a negative correlation was observed between evenness and the abundance of top clones with IGG. In contrast, positive correlations were found with the Gini index, Gini-Simpson index, and the IGG signature. All these metrics indicate that IGG-high tumors exhibit greater clonal diversity, with varying representation and distribution across both B and T cell populations.

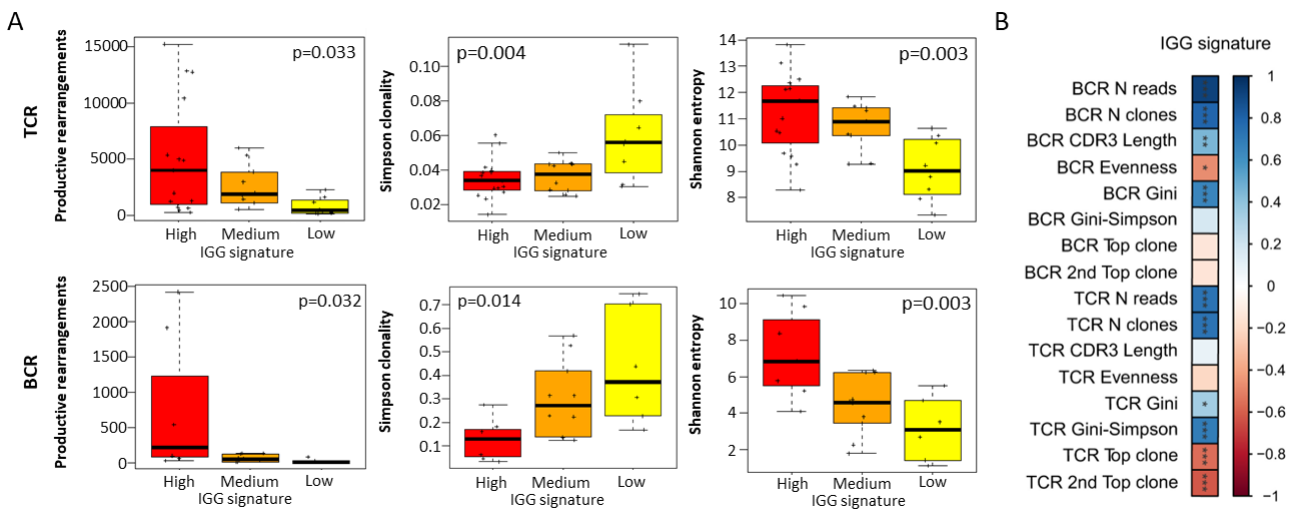


Figure 19. Association between IGG signature and BCR/TCR clonality. A) Comparison of TCR (top) and BCR (bottom) repertoire diversity metrics across IGG-high, IGG-medium, and IGG-low tumors, as assessed by the immunoSEQ assay. For TCR (n = 15 high, 10 medium, 8 low), and BCR (n = 7 high, 10 medium, 6 low) samples, the following parameters are shown: number of productive rearrangements (left), Simpson clonality index (center), and Shannon entropy (right). Overall, tumors with high IGG signature showed increased numbers of productive TCR and BCR rearrangements, lower clonality, and higher diversity (Shannon entropy), whereas tumors with low IGG signature displayed fewer

rearrangements, higher clonality, and reduced diversity. B) Pearson correlation analysis between BCR and TCR clonality measures and IGG levels in the HER2-positive BC CALGB-40601 cohort (n = 264), based on bulk RNA-seq data. ***P < 0.001, **P < 0.01, *P < 0.05.

5. Correlation between IGG, longevity, and B/T-cell clonality

Previous analyses showed that IGG-high tumors are more inflamed, enriched in TLS or TLS-like biological processes, and display greater B- and T-cell polyclonality. In addition, prior studies have linked the IGG signature with prognosis in BC^{19,63,64}. Building on this, we hypothesized that the IGG signature might also capture overall immune fitness, extending its significance beyond recurrence risk to long-term survival in patients who are effectively cured. To test this, we investigated the association between the IGG signature and OS in patients with early-stage BC who had not experienced a relapse, a condition we defined as ‘longevity’ (**Figure 20 A**). Using comprehensive gene expression and clinical data from three independent cohorts of 9,638 patients with early-stage BC (including 1,241 HER2-positive cases) and long-term follow-up (METABRIC¹⁵⁵, SCAN-B^{157,158}, and TCGA¹⁵⁶), we found a significant association between IGG expression and OS without recurrence. This association was observed both when treating recurrences as competing events (**Figure 20 B**) and when excluding recurrences entirely independently from BC subtype, tumor stage, or nodal status. Notably, the association persisted among patients at very low risk of relapse and those with >5 years of follow-up without documented recurrence. These findings suggest that the IGG signature may reflect systemic immune health contributing to patient longevity.

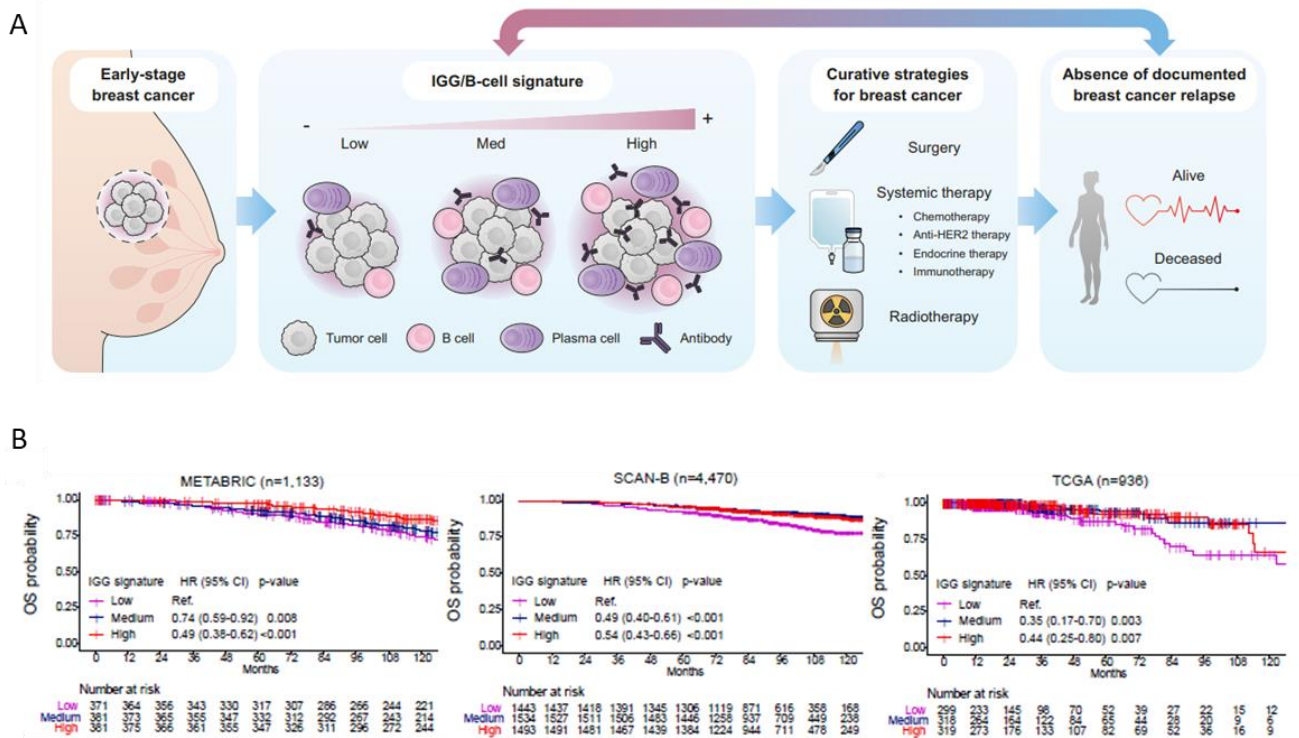


Figure 20. Association of IGG expression with OS in BC survivors without a documented recurrence. A) Schematic representation of the longevity. B) Kaplan-Meier plots with censored recurrences illustrating the association of IGG expression with OS in all patients from METABRIC (n = 1,133), SCAN-B (n = 4,470), and TCGA datasets (n = 936).

6. Association of IGG, PAM50 risk of recurrence and longevity

The PAM50 risk of recurrence (ROR) score is a well-established prognostic tool in early-stage BC, primarily reflecting tumor-intrinsic features such as proliferation and luminal biology. Given its widespread use, we asked whether ROR also predicts longevity, similar to our analysis of the IGG signature. To this end, we analyzed the relationship between the PAM50 ROR score and the IGG signature in the TCGA and METABRIC datasets. Differences in PAM50 ROR distribution were observed across IGG groups, showing that IGG-high tumors are enriched in both ROR-high and ROR-low categories, with a lower proportion of ROR-medium, particularly within HR+/HER2 BC (**Figure 21 A**). From a longevity perspective, the PAM50 ROR score was not significantly associated with OS in recurrence-free BC survivors (**Figure 21 B**). In contrast, the IGG signature remained independently associated with survival in this population, even after adjusting for ROR (**Table 4**).

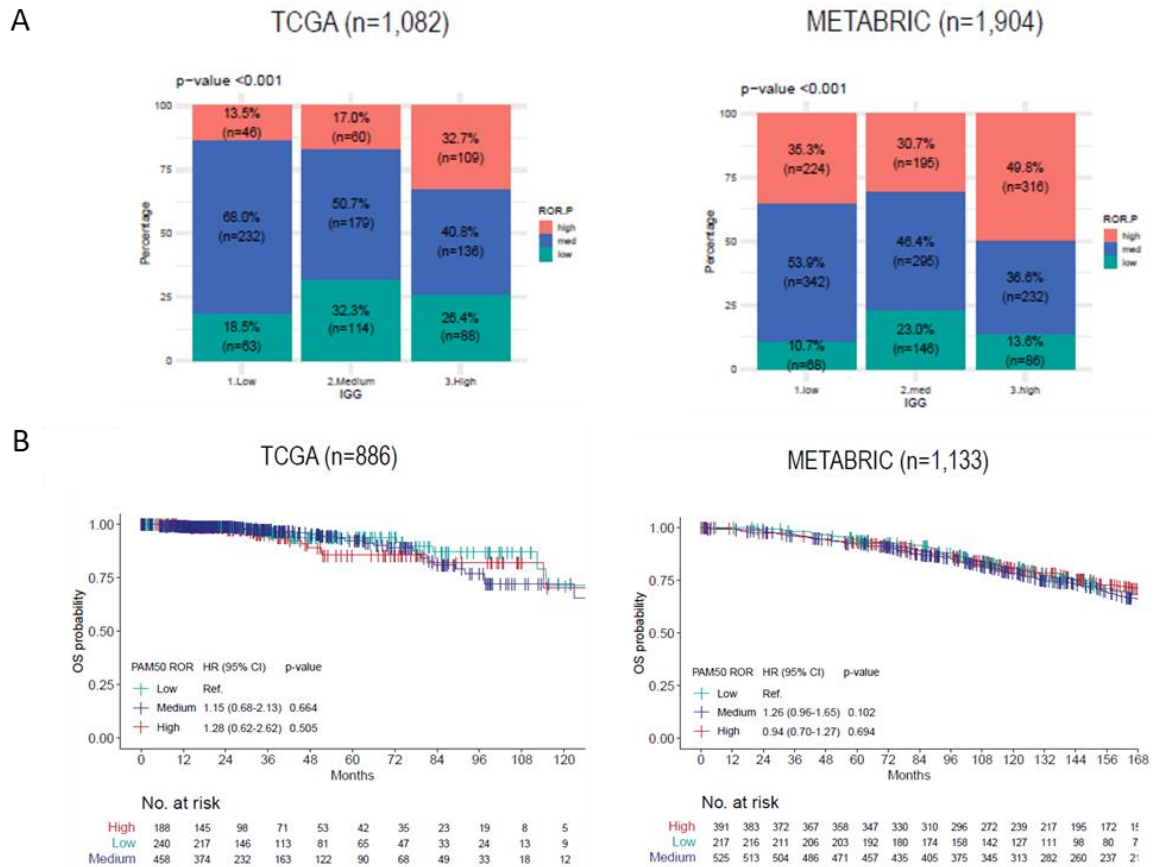


Figure 21. Association between PAM50 ROR and IGG expression and OS in BC survivors. A) Distribution of PAM50 ROR across IGG groups in all patients from TCGA (n=1,082) and METABRIC (N=1,904). B) Kaplan-Meier plots with censored recurrences illustrating the association between PAM50 ROR score and OS in TCGA (n = 886) and METABRIC datasets (n = 1,133).

TCGA	Univariate		Multivariable	
	HR (95%CI)	p-value	HR (95%CI)	p-value
ROR-low	Ref.		Ref.	
ROR-medium	1.15 (0.68-2.13)	0.664	1.47 (0.71-3.07)	0.300
ROR-high	1.28 (0.62-2.62)	0.505	0.94 (0.50-1.77)	0.850
IGG-low	Ref.		Ref.	
IGG-medium	0.35 (0.17-0.70)	0.003	0.34 (0.17-0.68)	0.003
IGG-high	0.44 (0.25-0.80)	0.007	0.39 (0.20-0.73)	0.004
METABRIC	Univariate		Multivariable	
	HR (95%CI)	p-value	HR (95%CI)	p-value
ROR-low	Ref.		Ref.	
ROR-medium	1.26 (0.96-1.65)	0.102	1.21 (0.92-1.60)	0.169
ROR-high	0.94 (0.70-1.27)	0.694	1.02 (0.76-1.39)	0.873
IGG-low	Ref.		Ref.	
IGG-medium	0.74 (0.59-0.92)	0.008	0.76 (0.60-0.95)	0.016
IGG-high	0.49	<0.001	0.50 (0.39-0.64)	<0.001

Table 4. Association of PAM50 ROR and IGG signature with overall survival in patients without relapse.

7. Linking IGG, age and BC subtype

Because immune function naturally declines with aging, we examined whether the IGG signature is influenced by patient age and whether its association with longevity is age-dependent. A weak negative correlation between the IGG signature and age was observed in all cohorts studied (**Figure 22 A, B**). Nevertheless, the IGG signature was significantly associated with OS without relapse in patients diagnosed between 41 and 70 years of age, who represented 66.7-72.9% of the cohort (**Figure 22 C**).

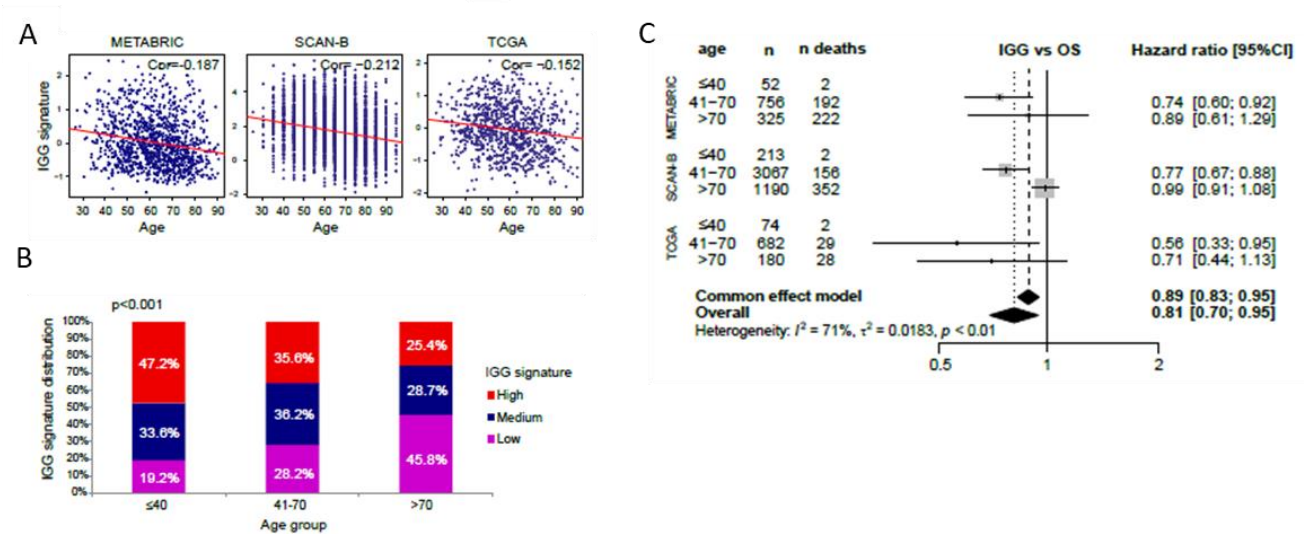


Figure 22. Relationship between the 14-gene IGG signature and patient age. A) Correlation analysis between the IGG signature and age in all patients without documented BC recurrence during follow-up in METABRIC (n = 1133), SCAN-B (n = 4470), and TCGA datasets (n = 936). B) Distribution of the IGG signature (low, med, high) across age groups in all patients without documented BC recurrence. Chi-square P value <0.001. C) Forest plot presenting the association of the IGG signature with OS in all patients without documented BC recurrence across different age groups, and in the pooled analysis of the three datasets (in bold).

Across BC subtypes, the IGG expression was higher in HER2-positive and TNBC compared to HR+/HER2- (**Figure 23 A**); however, 26.2% of HR+/HER2- tumors also exhibited high IGG expression. Finally, IGG expression decreased with age across BC subtypes, especially in patients >70 years with HR+/HER2- disease (**Figure 23 B**). Importantly, IGG levels assessed in normal breast tissue showed no association with age (**Figure 23 C**).

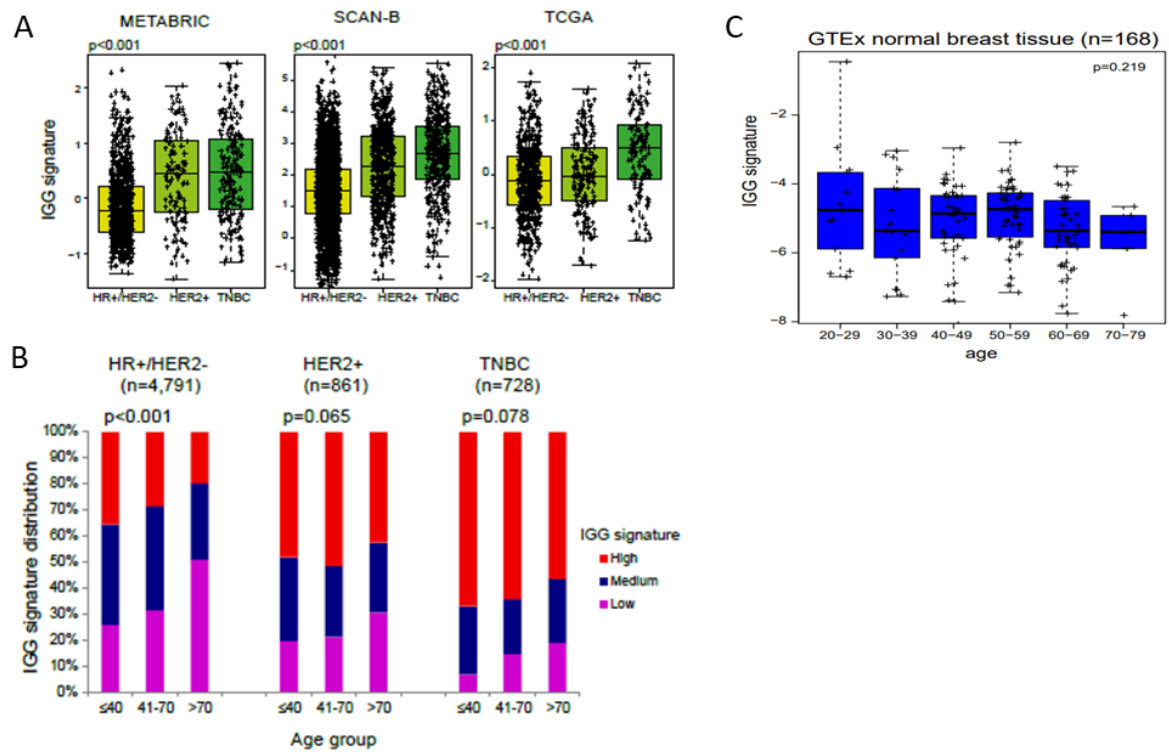


Figure 23. IGG expression across BC subtypes and normal breast tissue. A) IGG signature expression across BC clinical subtypes in all patients without documented BC recurrence. B) Distribution of the IGG signature (low, med, high) and age groups, stratified by BC subtype in all patients without documented BC recurrence. C) The relationship between IGG signature and age assessed in normal breast tissue.

8. Interplay of core IGG genes, longevity and B/T cell clonality

To determine whether the association with longevity was driven by the IGG signature as a whole or by specific genes within it, we next assessed the contribution of individual IGG genes. Seven genes of the 14 IGG genes (50.0%) —*TNFRSF17* (*BCMA*), *IL2RG*, *POU2AF1*, *CD27*, *IGJ* (*JCHAIN*), *CD79A*, and *PIM2*—collectively defining the CIGGs, showed a statistically significant association with longevity across all three datasets (**Figure 24 A**). A moderate correlation coefficient (Pearson $r = 0.63$) among the CIGGs suggested that they may track slightly different immune components (**Figure 24 B**). This raised the possibility that combining CIGGs could capture complementary signals and thereby improve their association with longevity.

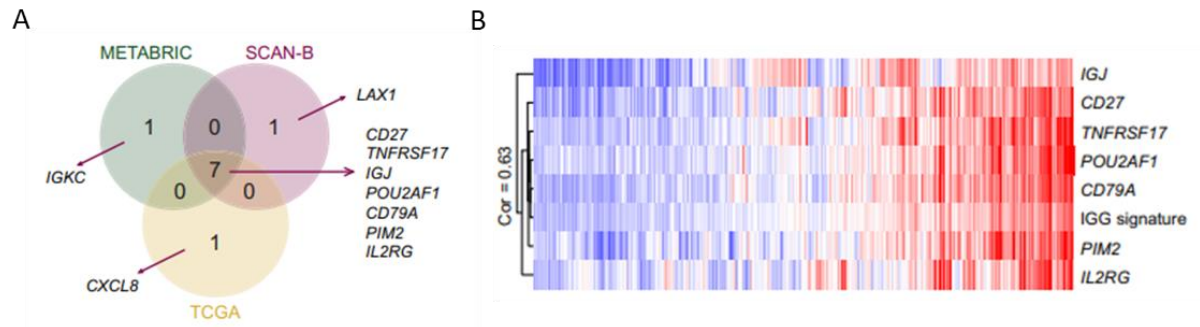


Figure 24. Identification of CIGGs related to longevity in patients with BC without documented recurrence. A) Venn diagram of the significant individual genes associated with longevity in univariate Cox regression models in METABRIC (n = 1133), SCAN-B (n = 4470), and TCGA datasets (n = 936). B) Expression of seven CIGGs and IGG signatures in the METABRIC dataset.

To evaluate if the combination of two CIGGs increases the ability to predict longevity over a single CIGG, we added the log base 2 expression values of two CIGGs. The combination of the two CIGGs into a single score provided a better association with longevity, when compared with single CIGG expression ($P < 0.001$) (**Figure 25 A**). In this context, the chi-square statistic from the likelihood ratio test in a Cox model reflects the overall improvement in model fit, with higher values indicating that the variable (or combination of variables) explains more of the variation in survival outcomes. Overall, 21 CIGG combinations increased the ability to predict longevity (**Figure 25 B**), and the immunoglobulin J chain gene (i.e. IGJ, also known as JCHAIN) and the interleukin 2 receptor subunit gamma gene (i.e. IL2RG) emerged as one of the top gene combinations.

We further examined the relationship between individual CIGGs—or any pairwise combinations—and BCR/TCR Shannon entropy. The combination of two CIGGs had similar prediction of BCR and TCR Shannon entropy as the IGG signature (**Figure 25 C**). Overall, these findings highlight the potential of the IGG signature and pairwise CIGG combinations derived from bulk RNA sequencing to capture effective adaptive B-cell and T-cell immune responses.

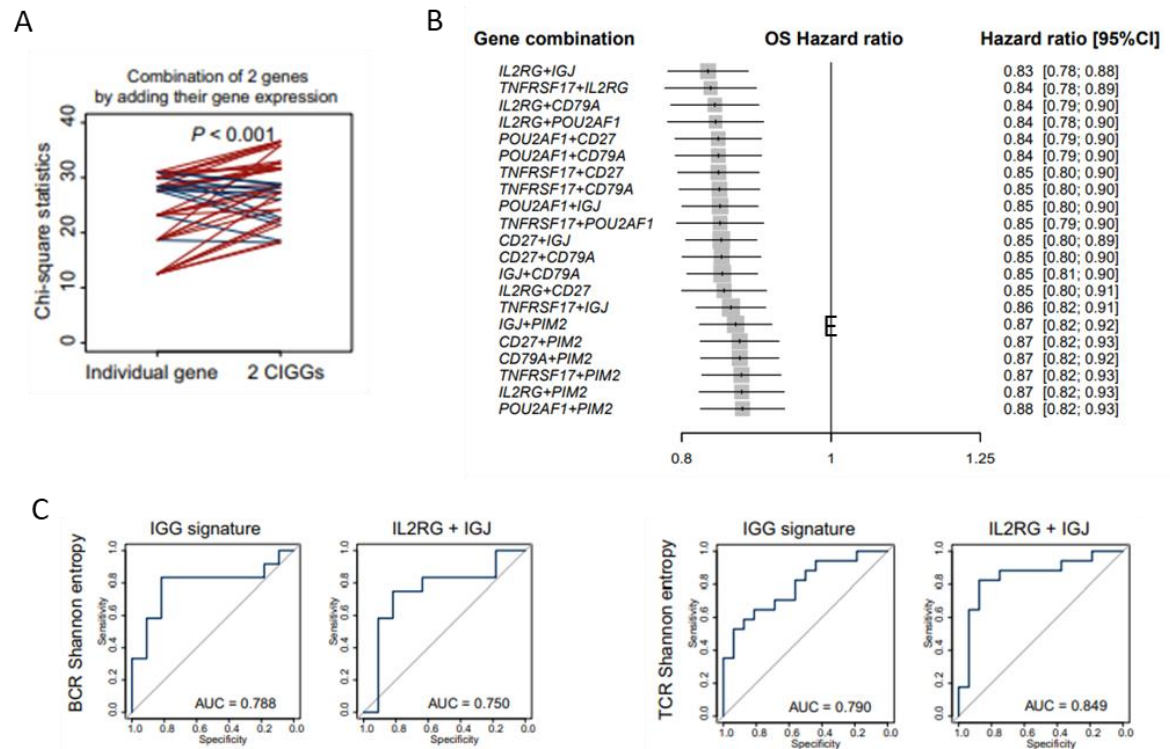
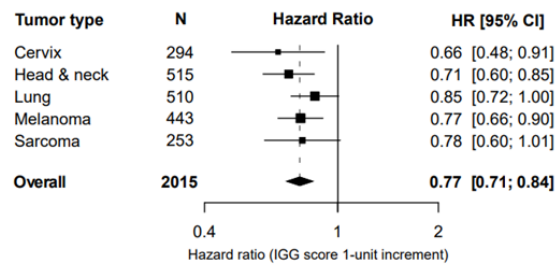


Figure 25. CIGGs gene combination and association between IGG and BCR/TCR clonality. A) Amount of variation explained in the prognosis of the expression of one single CIGG or the expression of the combination of two CIGGs (i.e. addition of the log base 2 expression values) as defined by chi-square statistics obtained from likelihood ratio testing. B) Association of 21 CIGG combinations and longevity C) ROC AUCs of IGG signature and the combination of the two CIGGs IL2RG + IGJ to predict BCR Shannon entropy across (left) and TCR Shannon entropy (right).

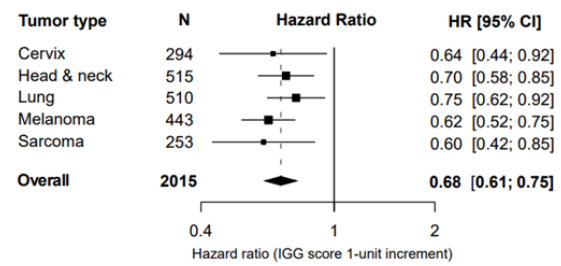
9. IGG and longevity in other cancer types

In the pan-cancer TCGA dataset, the IGG signature has been previously reported to be associated with OS in several tumor types, including cervical cancer (n = 294), head and neck cancer (n = 515), lung adenocarcinoma (n = 510), melanoma (n = 443), and sarcoma (n = 253)¹⁶⁰. Building on these findings, we specifically investigated whether the IGG signature was also associated with longevity in these cancer-types. Longevity was defined as OS in patients who remained recurrence-free. Consistent with the results observed in BC, the IGG signature was significantly associated with OS independent of relapse in a combined patient-level meta-analysis. This association was observed across the analyzed cancer types, with the exception of cervical cancer (Figure 26).

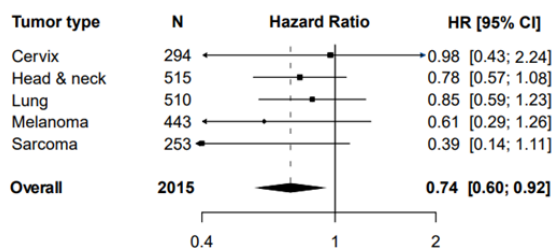
a Disease-free survival



b Overall survival



c Survival without recurrence (censoring recurrences)



d Survival without recurrence (excluding recurrences)

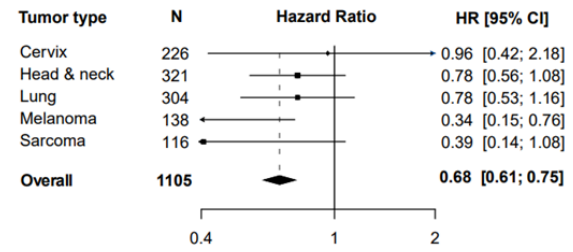


Figure 26. IGG and longevity across cancer types. Association of the IGG signature with disease-free survival (A), overall survival-OS- (B), OS without recurrence by censoring recurrences (C) and OS excluding recurrences (D) across different tumor types.

STUDY 2

CAR-T PROJECT

Optimization of ARI-HER2 ex vivo expansion

1. Characterization of the HER2-BBz CAR-T cells expansion process using different concentrations of dasatinib

One challenge encountered during the expansion of certain CAR-T cell products is that, when highly expressed on the T cell membrane, CARs can aggregate and induce tonic signaling even in the absence of antigen¹⁶¹. Tonic signaling significantly contributes to reduced persistence and functionality. This effect is particularly problematic when CAR constructs include the 4-1BB intracellular domain, as tonic signaling combined with initial CD3/CD28 activation can trigger T cell apoptosis within the first five days, thereby impairing proper expansion. To address this issue and ensure the generation of sufficient CAR-T cells after expansion, we tested a pharmacological inhibitor to suppress CAR signaling. The first approach evaluated was the addition of dasatinib to T cell culture, a compound previously reported to act as a pharmacological on/off switch for CAR-T cell signaling^{162,163}. Dasatinib is a tyrosine kinase inhibitor that blocks the phosphorylation of the CD3-z chain -associated with the protein kinase 70kDa (ZAP70)- by inhibiting the lymphocyte-specific protein tyrosine kinase (LCK). This mechanism effectively prevents signaling through both the CAR and the TCR (**Figure 9**).

During the early phase of T-cell expansion, dasatinib was added into the T cell culture on days 3 and 4 following CD3/CD28 T-cell activation (**Figure 27 A**). Treatment with dasatinib showed a dose-dependent enhancement of T cell expansion (from 0 to 1000 nM), as evidenced by increased population doubling (**Figures 27 B–C**). Notably, dasatinib also helped prevent the loss of CAR expression on the cell membrane during expansion (**Figure 27 D**). This effect was not due to increased CAR transduction, as dasatinib had no impact on the transduction efficiency itself (**Figure 27 E**). Furthermore, we demonstrated that supplementing the culture with dasatinib reduces 4-1BB-induced apoptosis (**Figure 27 F**). A minimum concentration of 50 nM was sufficient to achieve CAR-T cell expansion and reduced apoptosis levels comparable to those of control T cells. Beyond this threshold, a plateau effect was observed, with no

further enhancement in expansion or additional reduction in apoptosis at higher concentrations (**Figure 27 B and F**).

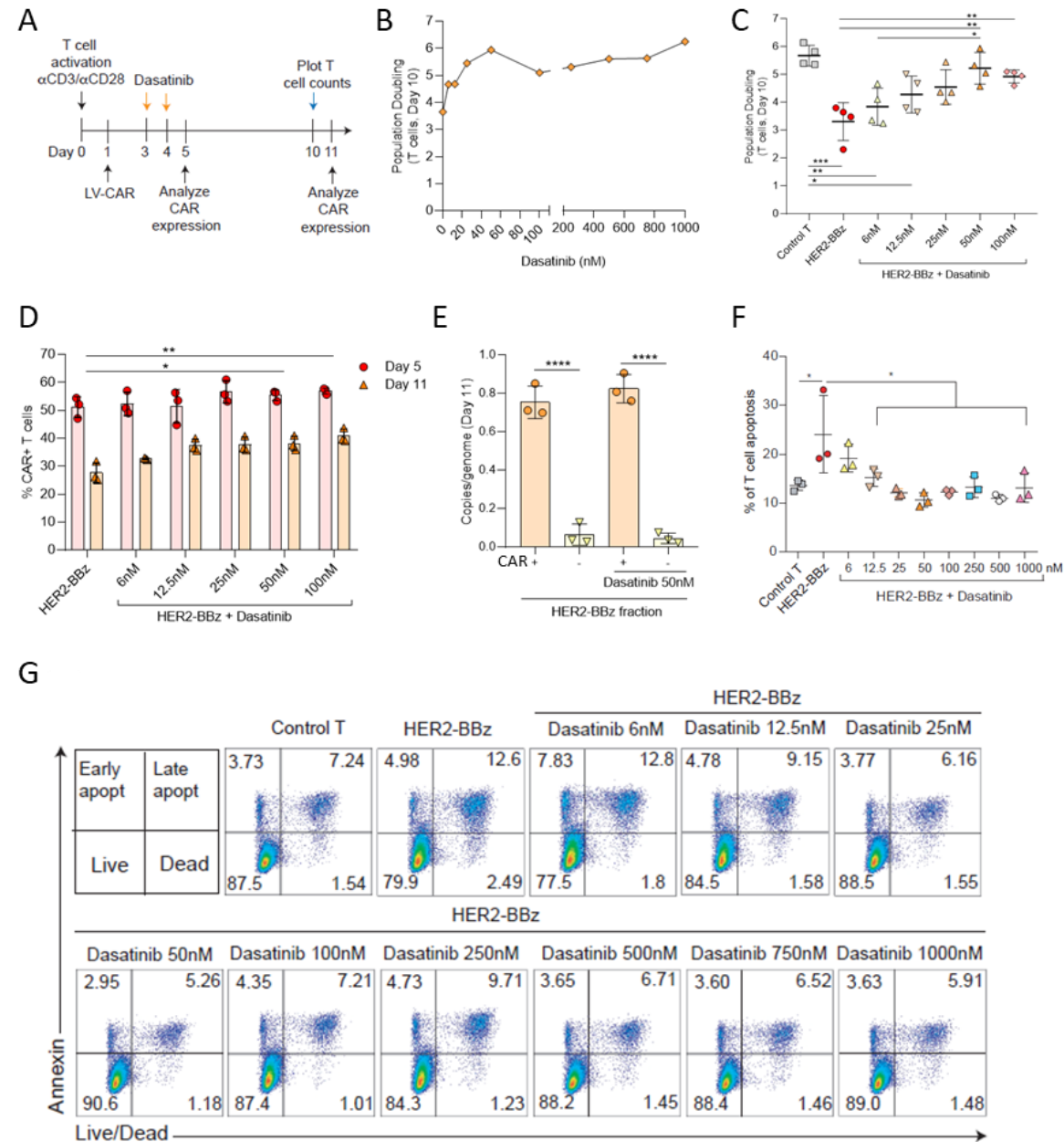


Figure 27. Addition of dasatinib during the ex-vivo expansion of HER2-BBz CAR-T cells improve T cell growth and reduce apoptosis. A) Schematic representation of the T cell expansion protocol. Dasatinib was added into the T cell culture on days 3 and 4. B, C) HER2-BBz CAR-T cells were expanded in the absence or presence of increasing doses of dasatinib. Population doublings at day 10 after T cell activation are shown. B) a dose-response curve (from 0-1000nM dasatinib) is shown for one normal donor (ND). A plateau on population doublings was reached at a dose of 50nM. In C) Population doublings of CAR-T cells (n=4 ND) with increasing doses of dasatinib (0-100nM) are compared to control non-transduced T cells and HER2-BBz CAR in absence of dasatinib. D) The percentage of T cells expressing the HER2-BBz CAR in their membrane was analyzed at day 5 and 11 after T cell activation (n=3

ND). E) The copies per genome in positive and negative fraction of CAR in presence of absence of dasatinib was analyzed by RT-PCR at day 11 after T cell activation (n= 3ND). CAR expression in sorted fractions based on surface CAR expression, with or without dasatinib treatment, was quantified by RT-qPCR on day 11 post T cell activation (n = 3 ND). F, G) Activation-induced cell death in CAR-T cells or control T cells from different ND (n=3) was analyzed by flow cytometry on day 6 after T cell activation by staining T cells with annexin and live/dead dye. F) The percentage of cell apoptosis was calculated by summing the population of cells positive for annexin and live/dead staining. G) Plots show the percentage of dead and apoptotic cells within the T cell population.

2. The influence of other drugs on the CAR-T expansion process

In CARs containing 4-1BB costimulatory domains, this phenomenon leads to NF- κ B activation via the TRAF2/IKK pathway, resulting in elevated expression of pro-apoptotic molecules such as FAS and FASL. Sustained signaling also activates the PI3K/AKT/mTOR axis, which induces metabolic stress and promotes T cell exhaustion (**Figure 9**). Collectively, these aberrant signaling events impair CAR-T cell expansion and survival.

To address this, we investigated the pharmacological inhibition of key components within these signaling networks, including AKT, PI3K, LFA-1, and FASL. Tyrosine kinase inhibitors (TKIs) have been shown to suppress T cell activation and may influence differentiation and exhaustion states.^{148,149} We therefore evaluated a range of TKI concentrations, including AKT and PI3K inhibitors (i), within a HER2-BBz CAR-T cell expansion protocol. In parallel, compounds targeting LFA-1 and FASL were also tested. All inhibitors were added on days 3 and 4 after T cell activation. The addition of AKTi (1 μ M), PI3Ki (1 μ M), or LFAi (2 μ g/mL) to the culture did not enhance CAR-T cell expansion. Furthermore, supplementation with FasLi (2 μ g/mL) or the use of higher concentrations of AKTi and PI3Ki resulted in a marked reduction of CAR-T cell expansion. Among the tested conditions, dasatinib 50nM was the only intervention that consistently improved CAR-T cell expansion (**Figure 28**).

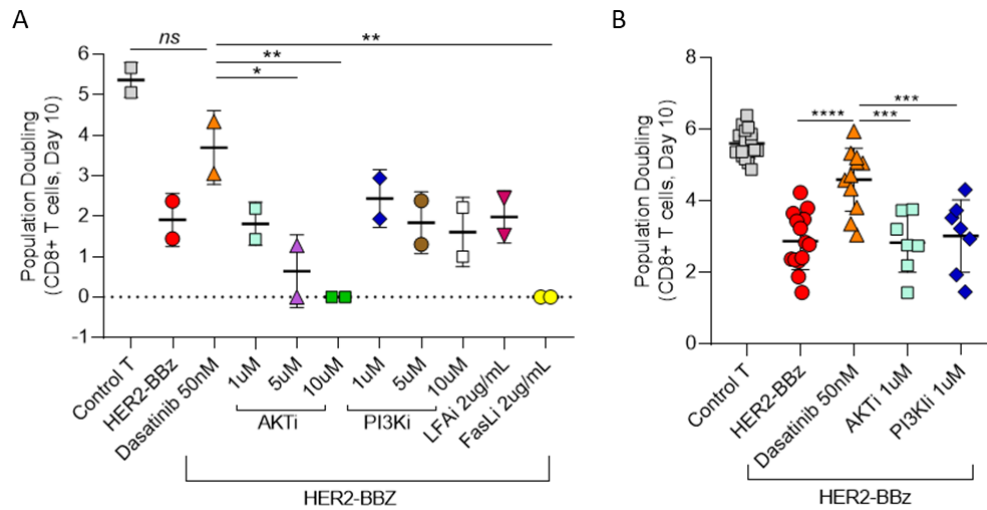


Figure 28. Dasatinib enhances CAR-T cell expansion compared to other pathway inhibitors. A,B) HER2-BBz CAR-T cells were expanded in the absence or presence of the indicated drugs at different doses in different ND. Population doublings at day 10 after T cell activation are shown. The addition of dasatinib 50nM but not the addition of inhibitors for AKT, PI3K, LFA or FasL could restore T cell population doublings.

3. HER2-BBz CAR-T cells expanded with dasatinib show effector functions *in vitro* and antitumoral efficacy *in vivo*

To ensure that the addition of dasatinib at different doses did not compromise the effector functions and antitumoral efficacy of HER2-BBz CAR-T cells, we next co-cultured CAR-T cells with the HER2-expressing ovarian cancer SKOV3 cell line. To evaluate cytokine production, CAR-T cells and tumor cells were co-cultured for 24 hours at 3:1 E:T ratio (**Figures 29 A-C**). HER2-BBz CAR-T cells expanded with dasatinib showed a similar (or slightly improved) IFN- γ and IL-2 secretion to CAR-T cells cultured without dasatinib. To assess the cytotoxic capacity of HER2-BBz CAR-T, CAR-T cells and tumor cells were co-culture at 3:1 E:T ratio, and real-time impedance measurements were used to quantify viable cell adherence. HER2-BBz CAR-T cells expanded in the presence of dasatinib displayed cytotoxic capacity comparable to that of cells expanded in its absence (**Figure 29 D**).

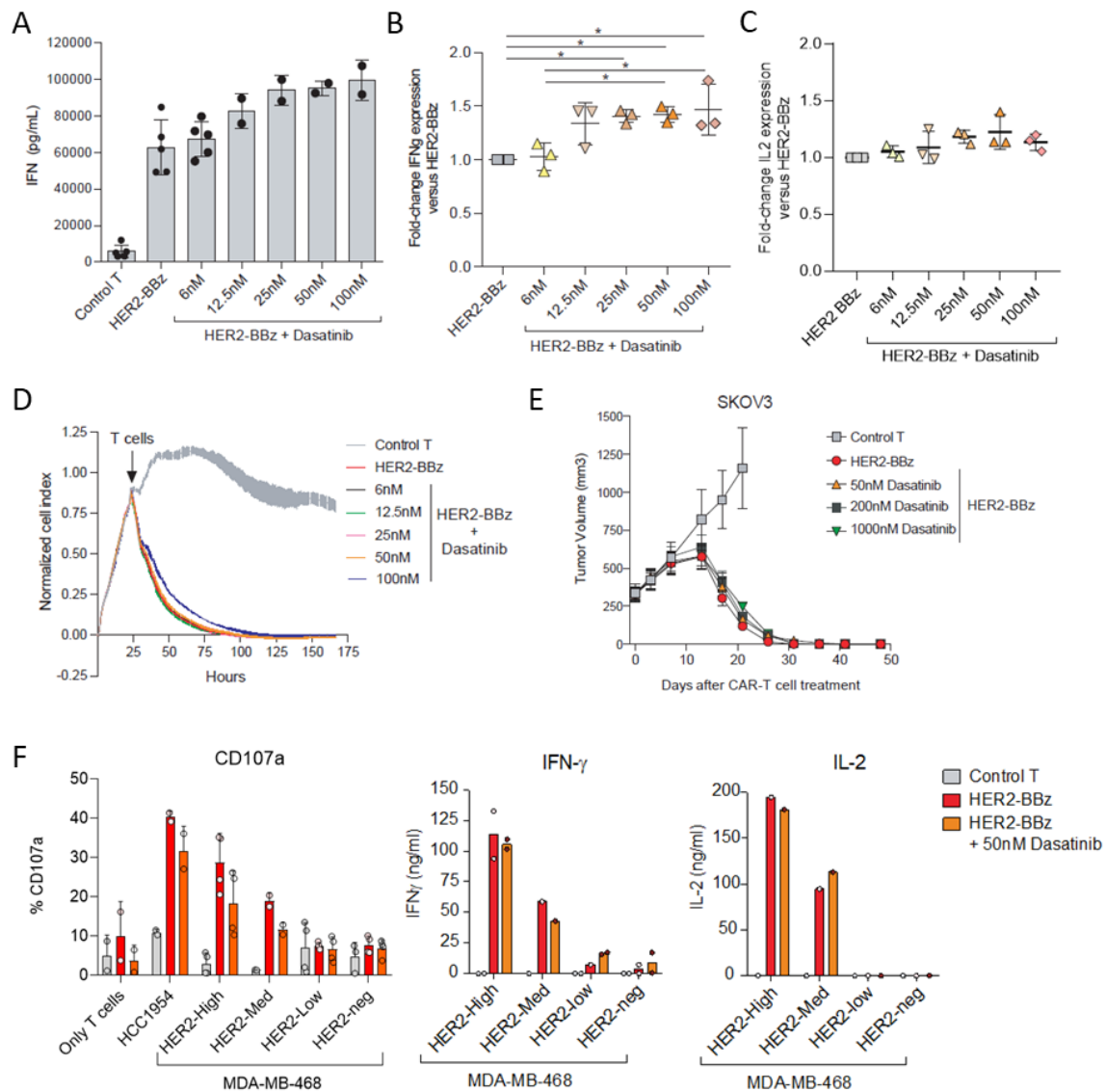


Figure 29. Addition of dasatinib during the ex-vivo expansion of HER2-BBz CAR-T cells does not decrease the therapeutic index of the HER2-BBz. HER2-BBz CAR-T cells were expanded in the absence or presence of increasing doses of dasatinib for 10 days and then used for functional assays or cryopreserved. A-C) CAR-T cells from different ND (n=2-5) were cocultured with HER2+ tumor cells (ovarian cancer, SKOV3) at an E:T=3:1. Supernatants were obtained 24 hours after coculture and cytokine production was analyzed by ELISA. IFN- γ concentrations (A) and fold-change IFN- γ (B) and IL-2 (C) expression are plotted. D) A real-time cytotoxicity assay (xCelligence) was used to evaluate the lysis of tumor cells when treated with control T cells or CAR-T cells at an E:T=3:1 over a 160-hour period. E) NSG mice bearing preestablished SKOV3 tumors were treated with one intravenous injection of 2×10^6 control-T or CAR-T cells. Tumor volume was monitored. The mean tumor volume $\pm$ SEM is shown. F) CAR-T cells were cocultured with tumor cells expressing different HER2 densities at an E:T=3:1. CD107a was analyzed by flow cytometry 4 hours after coculture. Supernatants were obtained 24 hours after coculture and IFN- γ and IL-2 production were analyzed by ELISA.

To assess the *in vivo* antitumor activity of the HER2-BBz CAR-T cells expanded with dasatinib, NSG mice were injected subcutaneously with $4\text{--}6 \times 10^6$ SKOV3 cells to generate an ovarian cancer mouse xenograft model. When the tumor size reached 350 mm^3 , animals were treated with an intravenous injection of 2×10^6 non-transduced control-T or HER2-BBz CAR-T cells expanded with or without dasatinib (**Figure 29 E**). HER2-BBz CAR-T cells cultured with dasatinib, even at the highest dose of 1000 nM , showed a similar antitumoral effect to CAR-T cells cultured without dasatinib, with all groups being able to eliminate all tumors by the end of the CAR-T cell treatment, compared to the control group.

Finally, we exploited a panel of target cells expressing different HER2 densities to evaluate whether the addition of dasatinib during culture could influence the safety profile of HER2-BBz CAR-T cells. The degranulation capacity (measured by CD170a expression), or the cytokine expression analysis (measured by IFN- γ and IL-2) showed identical profiles between both groups (**Figure 29 F**). HER2-BBz CAR-T cells expressed CD170a, IFN- γ and IL-2 when cocultured with cells that expressed high or medium levels of HER2. However, the activation profile of HER2-BBz CAR-T cells with cells expressing low or negative levels of HER2 was very similar to that of control cells lacking antitumoral specificity. In conclusion, these results suggest that the addition of dasatinib during the HER2-BBz CAR-T cells expansion would not increase the toxicity of the CAR-T therapy.

4. Characterization of the HER2-BBz CAR-T cells expansion process using different intervals for the addition of dasatinib

Zhang H. et al¹⁶⁴ demonstrated that the addition of 30 nM dasatinib, administered 5–7 days post-transduction within a 72-hour window in CD28/4-1BB CAR-T cells, modulates CAR-T cell differentiation, exhaustion, and apoptosis. Building on these findings, we investigated the *in vitro* expansion capacity of HER2-BBz CAR-T cells following dasatinib treatment at different timepoints of T-cell expansion. A comparative analysis was conducted by adding 50 nM dasatinib at different time intervals post-T cell activation:

from day 3 to day 4 (D3–D4), day 7 to day 9 (D7–D9)—mirroring the conditions described by Zhang et al.—and continuously from day 3 to day 9 (D3–D9).

Treatment with dasatinib during D3–D4 and D3–D9 significantly enhanced population doublings over time compared to untreated HER2-BBz CAR-T cells (**Figure 30 A**). Analysis of CAR expression further revealed that HER2-BBz CAR-T cells treated with dasatinib during D3–D4 and D3–D9 exhibited greater CAR surface expression than those treated during D7–D9 or left untreated (**Figure 30 B**).

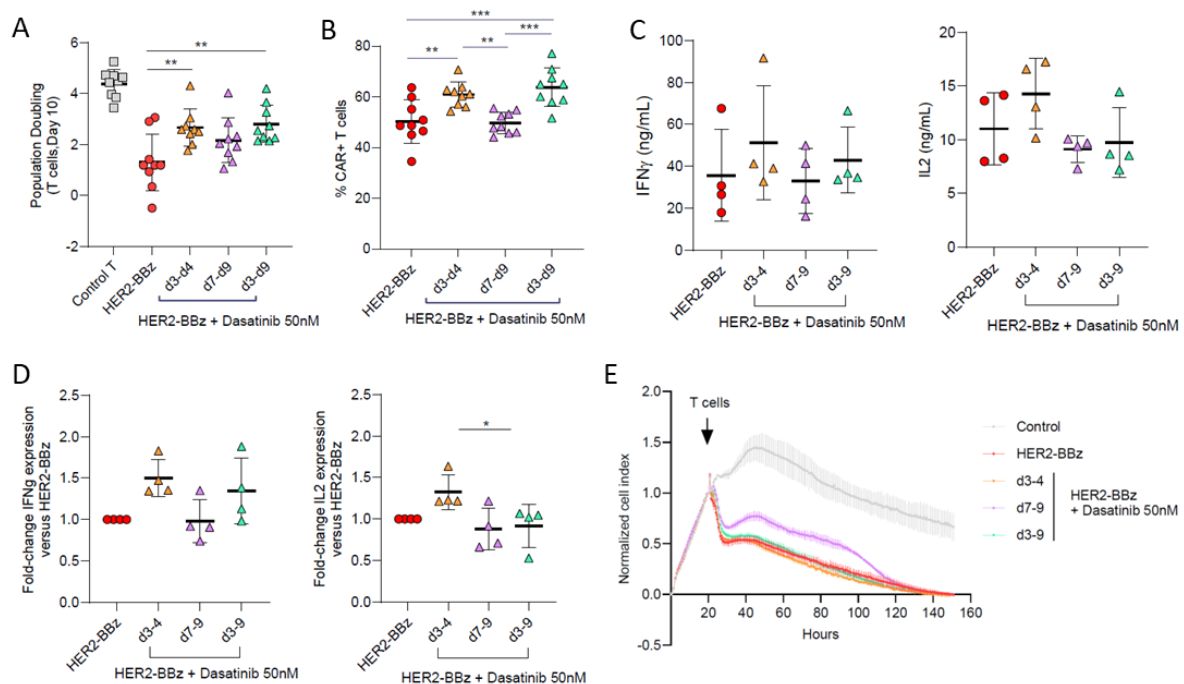


Figure 30. Short-term exposure to 50 nM dasatinib early after T cell activation (days 3–4) enhances HER2-BBz CAR-T cell expansion and CAR expression, while preserving cytotoxic function and modestly improving cytokine secretion. A-B) HER2-BBz CAR-T cells from different ND (n=9) were expanded in the presence of 50 nM dasatinib across different time intervals (day 3–4, day 7–9, and day 3–9). A) Population doublings measured on day 10 post-T cell activation. B) The percentage of T cells expressing the HER2-BBz CAR in their membrane was analyzed at day 8 after T cell activation. C-E) CAR-T cells from different ND (n=4) were cocultured with HER2+ tumor cells (ovarian cancer, SKOV3) at an E:T=3:1. C-D) Supernatants were obtained 24 hours after coculture and cytokine production was analyzed by ELISA. IFN-γ concentrations (C) and fold-change (D) IFN-γ and IL-2 expression are plotted. E) A real-time cytotoxicity assay (xCelligence) was used to evaluate the lysis of tumor cells when treated with control T cells or CAR-T cells at an E:T=3:1 over a 150-hour period.

To assess cytotoxic activity, HER2-BBz CAR-T cells expanded under each condition were co-cultured with HER2-positive SKOV3 ovarian cancer cells. Cytotoxicity and cytokine production were evaluated at an E:T ratio of 3:1 (**Figures 30 C–E**). HER2-BBz CAR-T cells

treated with dasatinib during D3–D4 displayed cytotoxic capacity comparable to that of untreated HER2-BBz CAR-T cells (**Figure 30 E**), while exhibiting a modestly enhanced cytokine secretion profile, as indicated by increased fold-changes in IFN- γ and IL-2 levels (**Figures 30 C–D**). The other treatment groups did not show statistically significant differences.

5. Analysis of ARI-HER2 transcriptomics in the presence or absence of dasatinib during ex-vivo expansion of non-clinical batches

To uncover the transcriptional changes underlying the effects of adding dasatinib on HER2-BBz CAR-T cells during ex-vivo expansion from non-clinical batches, we compared the transcriptomic profiles of UTD and HER2-BBz CAR-T cells cultured with or without dasatinib using the nCounter® CAR-T Characterization Gene Expression Panel (NanoString Technologies) (**Figure 31**). HER2-BBz CAR-T cells without dasatinib showed the highest number of differentially expressed genes (92) compared to UTD T cells, (**Figure 31 A left panel**). In contrast, the addition of dasatinib resulted in 68 differentially expressed genes (**Figure 31 A, middle panel**), suggesting that dasatinib treatment mitigates tonic signaling, reducing the transcriptomic differences between HER2-BBz CAR-T cells and control T cells. Among the upregulated genes in HER2-BBz CAR-T cells without dasatinib compared to UTD T cells, we identified T-cell activation-related genes such as effector cytokines or chemokines and apoptosis-related genes. Of note, these apoptosis-related genes were downregulated when dasatinib was added to HER2-BBz CAR-T cells (**Figure 31 A, right panel**). We next performed gene enrichment analysis, which confirm the upregulation of the apoptotic pathway in HER2-BBz CAR-T cells without dasatinib (**Figure 31 B**), and its downregulation upon dasatinib treatment (**Figure 31 C**). These findings further demonstrate that the addition of dasatinib during the ex-vivo expansion of non-clinical batches of HER2-BBz CAR-T cells reduce the T cell apoptosis triggered by tonic signaling in 4-1BB containing CARs.

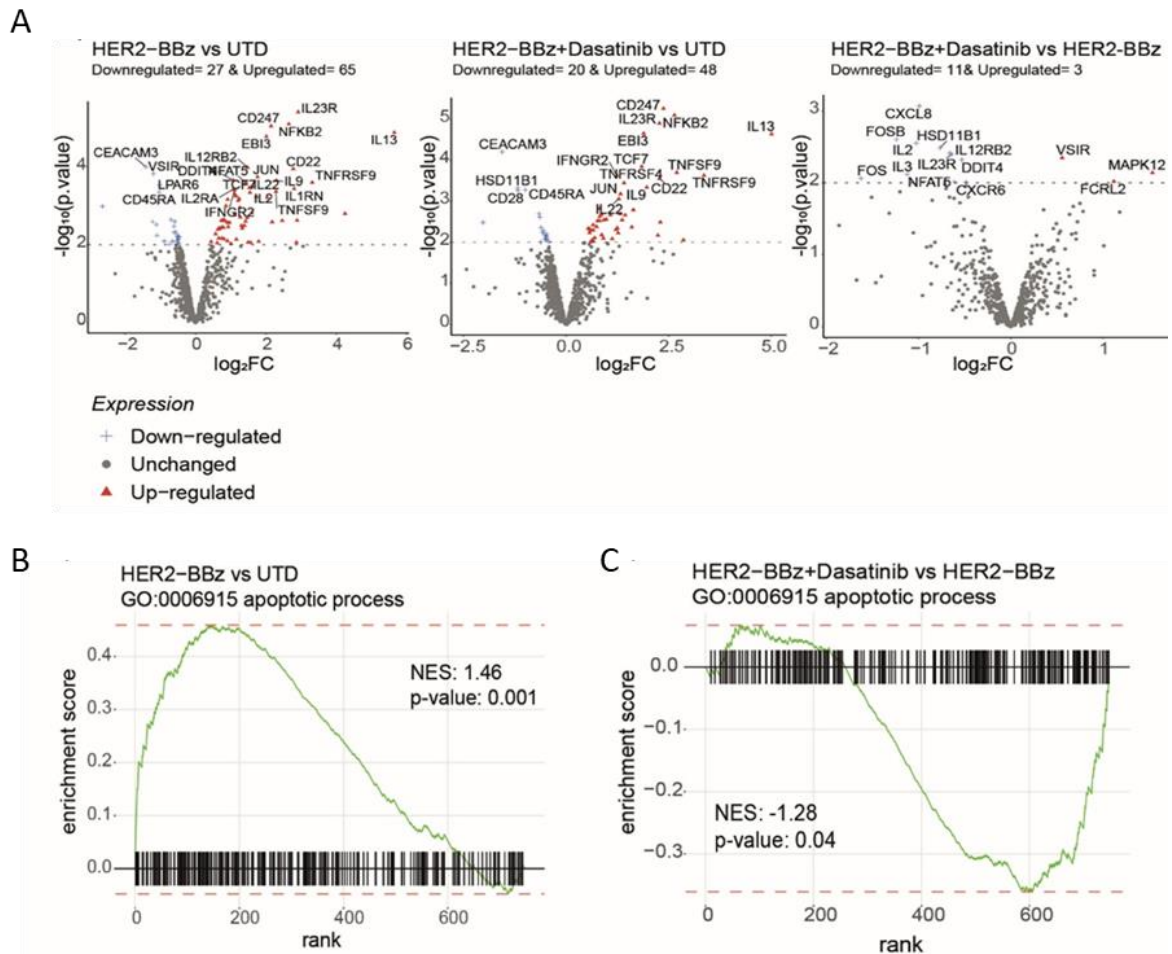


Figure 31. Transcriptomic analysis of HER2-BBz CAR-T cells with and without dasatinib during their ex-vivo expansion. A) Volcano plots of differential gene expression comparisons between HER2-BBz CAR-T cells without dasatinib cells and UTD T cells (left panel), HER2-BBz CAR-T cells with dasatinib and UTD T cells (middle panel), and HER2-BBz CAR-T cells with versus without dasatinib. Red triangles represent genes upregulated, and blue crosses represent genes downregulated in HER2-BBz CAR-T cells (left panel) and HER2-BBz CAR-T cells with dasatinib (middle and right panels). Grey dots represent genes not differentially expressed. Top significant differentially expressed genes are labelled. The horizontal dot line is at a p value of 0.01. B-C) Gene set enrichment analysis (GSEA) of the Gene Ontology apoptotic process pathway (GO:0006915) using significantly expressed genes in HER2-BBz CAR-T cells versus UTD T cells (B) and from HER2-BBz CAR-T cells with dasatinib versus without dasatinib (C). The differential expression analysis was done in R using the *limma* package v.3.58.1 and GSEA plots using the *fgsea* package v.1.28.0 and Gene Ontology Biological Process database.

6. The addition of dasatinib enhances T-cell across distinct CAR constructs

We have shown that the addition of 50 nM dasatinib on days 3 and 4 post-activation enhances the expansion of HER2-BBz CAR-T cells, increases CAR surface expression, and reduces apoptosis. To evaluate whether this effect is consistent across other CAR constructs, we tested the same conditions using a CAR targeting mesothelin (M11) incorporating the 4-1BB costimulatory domain. When 50 nM dasatinib was added during days 3 to 4 of the M11-BBz CAR-T cell expansion protocol, a similar pattern was observed: T-cell proliferation was enhanced (**Figure 32 A**), CAR surface expression increased (**Figure 32 B**), and apoptosis levels were reduced (**Figure 32 C**), compared to M11-BBz CAR-T cells cultured without dasatinib. These results suggest that early, short-term exposure to dasatinib may represent a broadly applicable strategy to enhance the expansion, viability, and CAR surface expression of CAR-T cells incorporating the 4-1BB costimulatory domain, regardless of the targeted antigen.

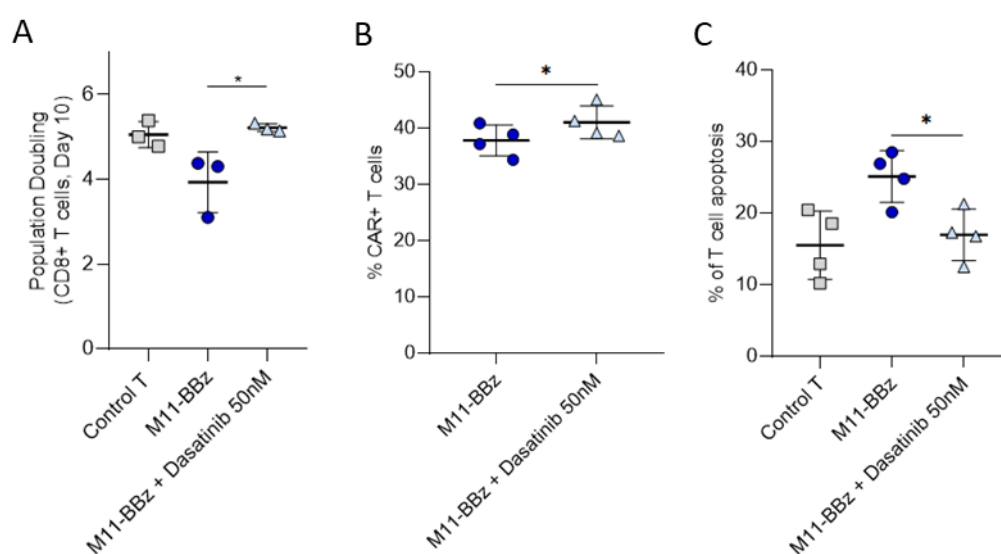


Figure 32. Addition of dasatinib during the ex-vivo expansion of Meso-BBz CAR-T cells improves T cell growth and CAR expression. T cells were genetically modified to express a CAR targeting mesothelin (Meso-BBz). The Meso-BBz CAR contains the M11 scFv targeting mesothelin in tandem with the CD8 hinge and transmembrane domains and the 4-1BB and CD3 intracellular domains. Meso-BBz CAR-T cells were expanded in the absence or presence of dasatinib (50nM). T cells were counted overtime. A) Population doublings at day 10 are shown (n=3 ND). B) The percentage of T cells expressing the Meso-BBz CAR in their membrane was analyzed on day 6 after T cell activation (n=4 ND). C) The percentage of apoptotic cells in CAR-T cells or control T cells was assessed by flow cytometry on day 6 after activation (n=4 ND). The percentage of cell apoptosis was calculated by summing the population of cells positive for annexin and live/dead staining.

7. HER2-BBz T cell expansion and functional characterization from breast cancer patients' PBMCs

Finally, to evaluate the potential clinical applicability, we prepared HER2-BBz non-clinical batches using peripheral blood mononuclear cells (PBMCs) derived from two patients with HER2-positive BC. T cells were initially isolated using the EasySep™ Human CD3 Positive Selection Kit, resulting in a comparable CD4/CD8 ratio across both patient samples (**Figure 33 A**). T cells were then transduced and expanded ex-vivo in the presence or absence of dasatinib following the previously described protocol.

HER2-CAR-T cells cultured with or without dasatinib demonstrated comparable CAR surface expression levels (**Figure 33 B**), cytokine secretion (**Figure 33 C**), and cytotoxic activity (**Figure 33 D**) upon antigen recognition in HER2+ cells. These findings suggest that HER2-BBz (ARI-HER2) can be successfully transduced into T cells derived from patients with HER2-positive BC, and that dasatinib does not impact CAR-T cell functionality.

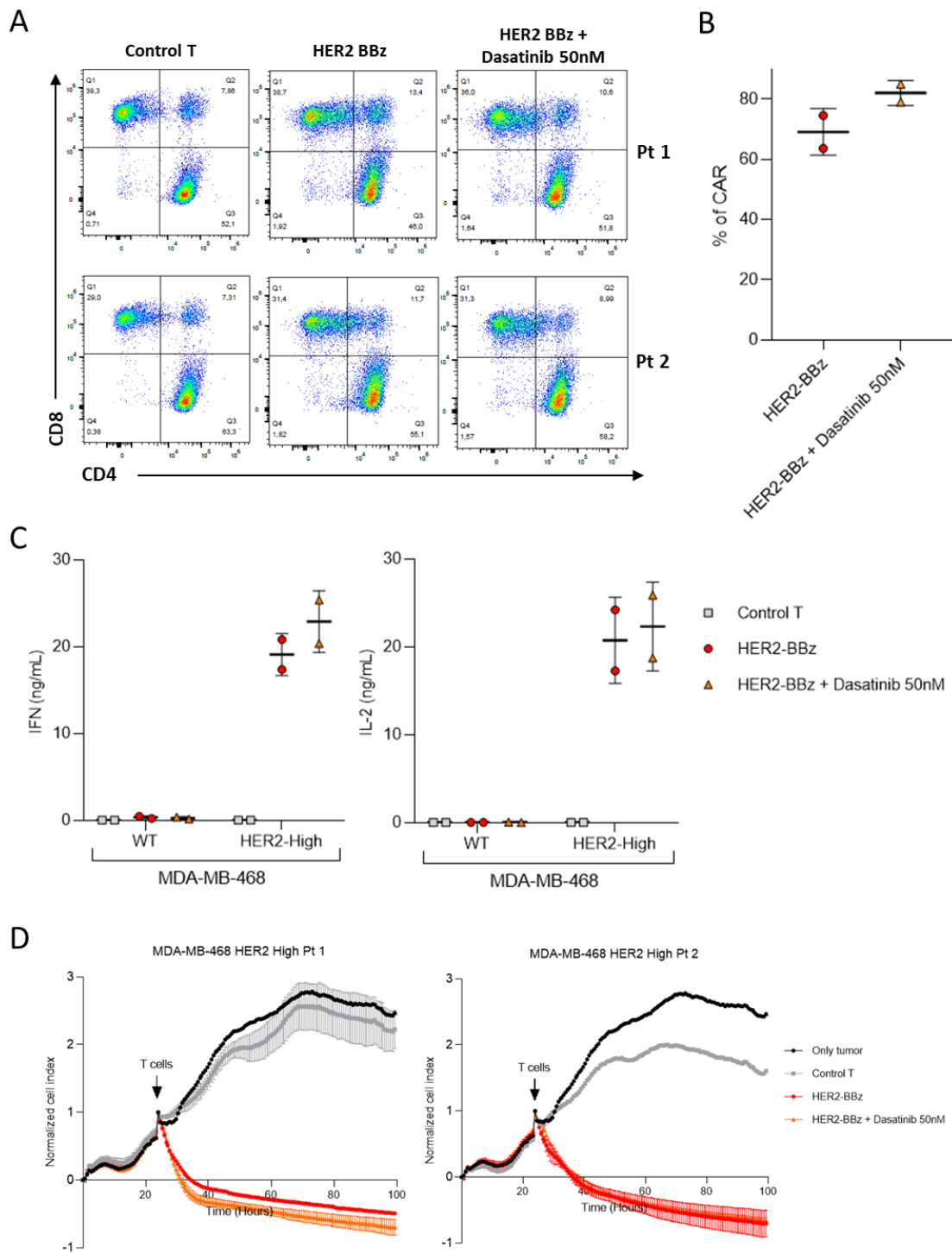


Figure 33. HER2-BBz can be efficiently generated using T cells from patients with HER2-positive BC. A) CD4/CD8 T cell ratio at the end of the expansion of CD3⁺ T cells from two HER2-positive BC patients (pt). B) Percentage of CAR expression in HER2-BBz cultured with or without dasatinib. C) Cytokine production by CAR-T cells (n = 2) following 24-hour co-culture with HER2-negative (MDA-MB-468 WT) or HER2-overexpressing (MDA-MB-468 High) tumor cells at an effector-to-target (E:T) ratio of 3:1. IFN- γ and IL-2 levels in supernatants were quantified by ELISA. Each dot represents an individual patient. D) Real-time cytotoxicity assay (xCELLigence) was used to evaluate the lysis of tumor cells when treated with control T cells or CAR-T cells at an E:T=3:1 over a 100-hour period.

Safety and toxicity profile of the CAR-T cell candidate

1. Comparison between HER2-BBz and high affinity third generation HER2 CAR-T cells targeting high and low HER2 densities in vitro

To better understand both the antitumor efficacy and the toxicity of HER2-BBz, we compared our CAR-T construct based on the 4D5.5 low affinity scFv with a HER2-targeting CAR-T cell product that had exhibited toxicity in early clinical trials¹²⁸. HER2-BBz and the anti-HER CAR, developed in 2010 at NIH, differ in two key aspects: the HER2-recognition affinity and the costimulatory domains. HER2-BBz is based on the 4D5.5 scFv that recognized HER2 with low-affinity (LA) (hereafter referred to as HER2-BBz LA) as previously described in this document and is a second-generation CAR with 4-1BB as costimulatory domain. The CAR from NIH contains the high-affinity (HA) 4D5 scFv derived from trastuzumab (referred to as HER2-28BBz HA NIH). HER2-28BBz HA is a third-generation CAR containing both the 4-1BB and the CD28 costimulatory domains within the CAR molecule. Additionally, HA CAR differs from HER2-BBz at the leader sequence and the hinge domain (**Figure 34 A**). The administration of a very high dose of modified T cells with the HER2-28BBz HA NIH resulted in the death of a patient due to potential activation of these cells in lung tissue.

In the following assays, we compared the therapeutic index of our clinical candidate HER2-BBz (the low affinity HER2 CAR) with the HER2 CAR developed at NIH at a preclinical level. We first assessed their cytokine secretion profile (IFN- γ and IL-2) after co-culture with different cells, including: the MDA-MB-468 tumor cells expressing different HER2 densities (high, low and negative), and a panel of primary cells expressing physiological HER2 levels.

First, we observed that both HER2-BBz and HER2-28BBz (HA NIH) CAR-T cells were activated at similar levels upon co-culture with tumor cells overexpressing HER2 (**Figures 34 B-C left panel**). Importantly, HER2-BBz did not get activated when cocultured with tumor cells expressing low or negative HER2 densities (**Figures 34 B-C**

right panel), while HER2-28BBz (HA NIH) CAR-T cells were still able to produce high levels of IFN- γ and IL-2 (more than 10 times compared to our candidate, HER2-BBz).

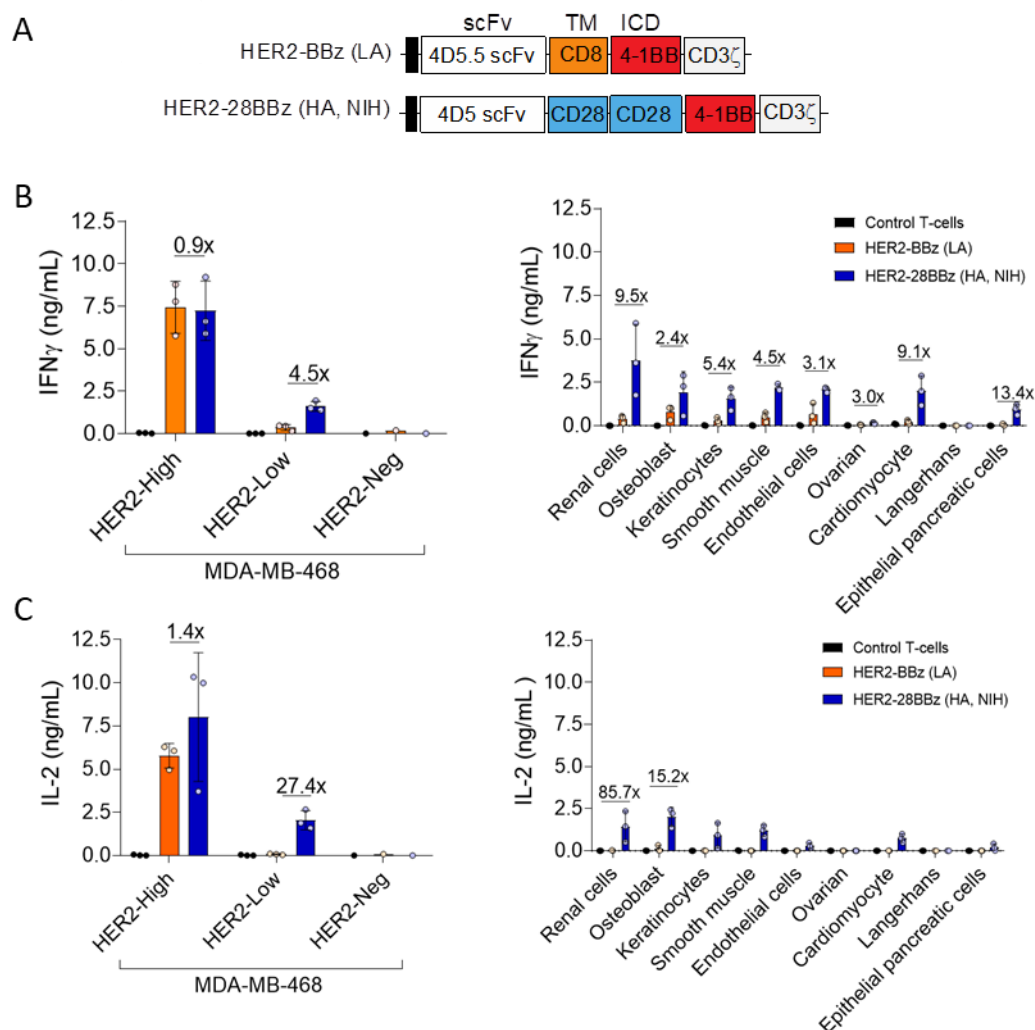


Figure 34. HER2-BBz CAR can produce cytokines in the presence of high HER2 densities but not in the presence of physiological levels of HER2. A) Schematic representation of the low-affinity CAR (HER2-BBz) and the high-affinity CAR (HER2-28BBz) used at the NIH clinical studies. B-C) The indicated CAR-T cells were cocultured with HER2 positive tumor cells (MDA-MB-468 with varying levels of HER2; left panels) or with normal cells (right panels) including renal cells, osteoblasts, keratinocytes, smooth muscle cells, endothelial cells, ovarian cells, cardiomyocytes, Langerhans cells and pancreatic cells at an E:T ratio of 3:1. Supernatants were collected 24 hours after coculture and cytokine levels of (B) IFN- γ and (C) IL-2 were measured by ELISA. The fold-change cytokine production by high-affinity CAR-T cells compared to low-affinity CAR-T cells is shown for each cell line. Each dot represents a different healthy donor.

To extend the above-mentioned studies, we assessed T-cell activation by surface CD107a expression (degranulation marker), and CAR-T cell mediated cytotoxicity (Figure 35).

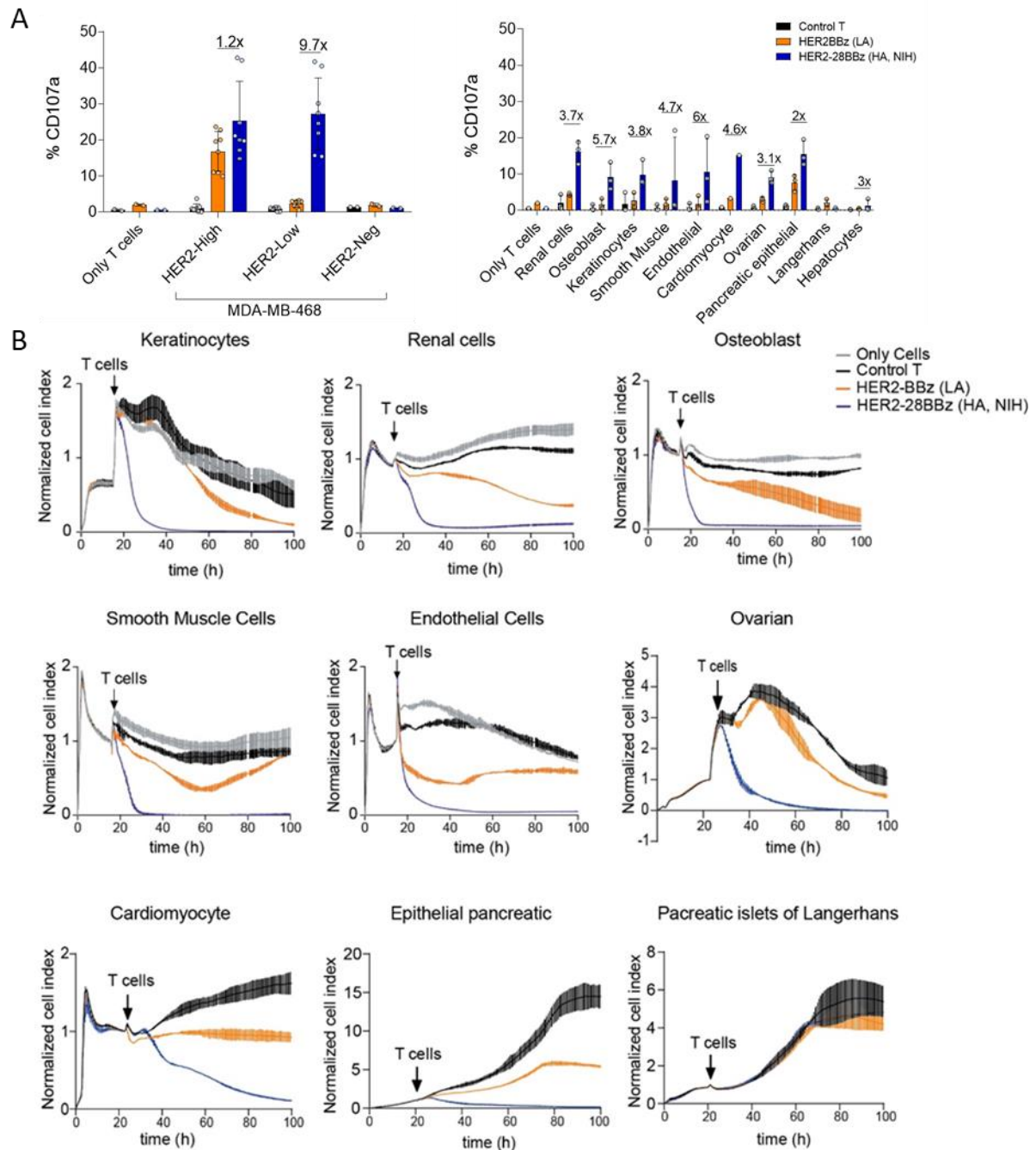


Figure 35. HER2-BBz CAR can degranulate and kill when cocultured with tumors that express high HER2 densities but not in the presence of physiological levels of HER2. A) Indicated CAR-T cells were cocultured with HER2 positive tumor cells or normal cells at an E:T=3:1. CD107a expression was analyzed by flow 4 hours after coculture. The fold-change CD107a expression in low affinity HER2-BBz CAR-T cells versus high affinity HER2-28BBz CAR-T cells is shown for each cell line. Each dot represents a different health donor. B) A real-time cytotoxicity assay (xCelligence) was used to evaluate the lysis of normal cells when treated with control T cells or CAR-T cells at an E:T=3:1 over a 100-hour period.

In line with previous observations, NIH CAR-T cells were capable of degranulating and inducing target-mediated killing after coculture with normal cells. In contrast, HER2-

BBz CAR-T cells displayed activity comparable to control T cells, failing to upregulate CD107a (**Figure 35 A**) or mediate cytotoxicity (**Figure 35 B**). Notably, to model worst-case scenarios, these experiments employed elevated E:T ratios (3:1), which exceed those expected in clinical settings.

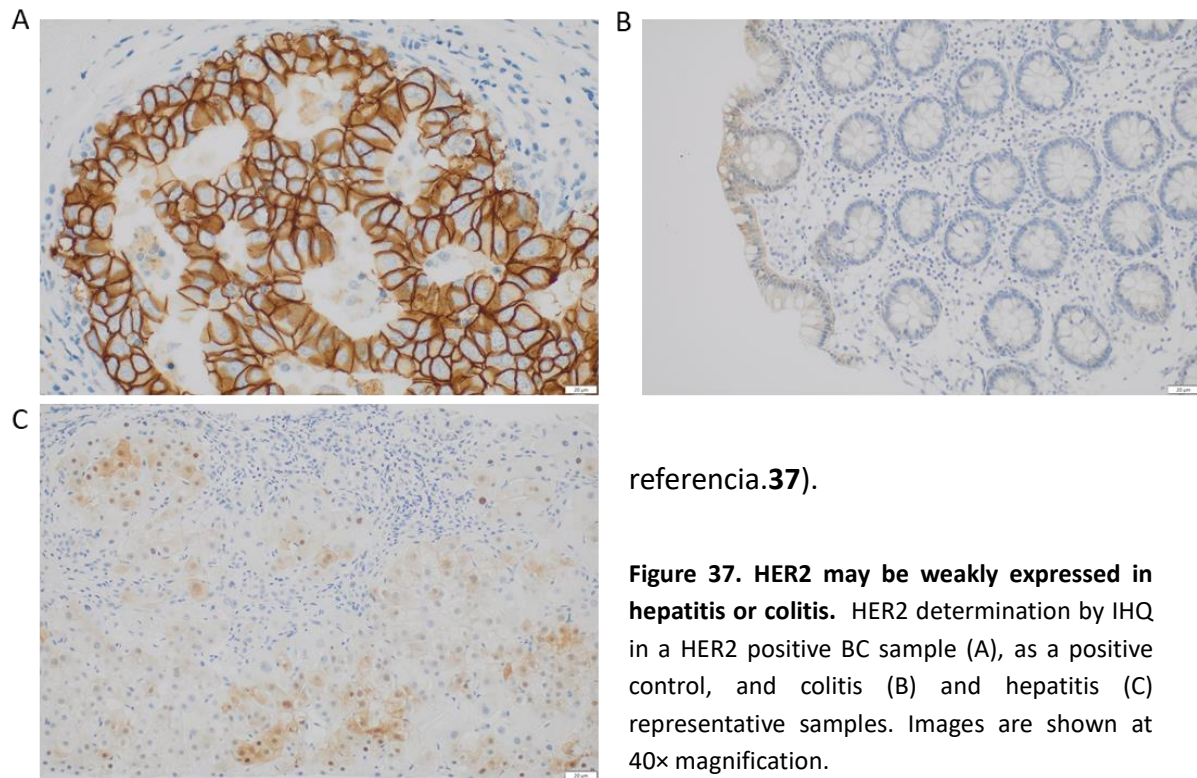
These results indicate that HER2-BBz CAR selectively eliminates tumor cells overexpressing HER2 while sparing healthy cells expressing physiological HER2 levels.

In a previous study conducted by our laboratory (*Marzal B thesis, Receptor antigénico quimérico en linfocitos T para el tratamiento del cancer de mama HER2+, 2023*), the *in vivo* ability of the HER2-BBz CAR to distinguish between high and physiological levels of HER2 in animal models was investigated. As part of the study, the high-affinity HER2-28BBz CAR developed by the NIH was also tested. The results suggest that the HER2-BBz CAR exhibits antitumor efficacy comparable to that of the HER2-28BBz CAR, but with reduced toxicity, thereby enhancing its therapeutic window.

2. Evaluation of HER2 expression in pathological non-tumoral conditions

In November 2022, a scientific advice meeting was held with the AEMPS to present our results and obtain their opinion regarding the preclinical development of a new product. During the discussion, toxicological concerns were raised about the potential overexpression of HER2 under pathological inflammatory conditions, such as colitis and hepatitis, in non-tumoral contexts. Concerns pointed that, as HER2-BBz CAR-T cells can potentially persist in the patient for months or years, they could induce future toxicities in case HER2 was overexpressed in pathological conditions. Therefore, it was important to assess: 1) whether HER2 levels were increased in inflammatory conditions and 2) whether these HER2 levels in case of positivity were comparable to those low levels observed physiologically in some healthy tissues or higher and therefore similar to that observed in tumor cells.

Therefore, we analyzed five inflammatory tissue samples from hepatitis and five from colitis from an in-house cohort. From the ten samples evaluated, two of them showed slightly expression of HER2 protein by IHC (¡Error! No se encuentra el origen de la



referencia.37).

Figure 37. HER2 may be weakly expressed in hepatitis or colitis. HER2 determination by IHC in a HER2 positive BC sample (A), as a positive control, and colitis (B) and hepatitis (C) representative samples. Images are shown at 40x magnification.

To further characterize this observation, *ERBB2* mRNA expression was analyzed in inflammatory tissue samples (n=10) and compared with HER2-positive (n=132) and HER2-negative (n=142) breast cancer FFPE samples. Additional comparisons were made with cell lines and tissues exhibiting varying HER2 expression levels, including HCC1954, T47D, and MDA-MB-468, as well as with normal cells such as keratinocytes, renal cells, osteoblasts, cardiomyocytes, pancreatic cells, ovarian cells, and Langerhans cells. The analysis was performed using a panel of 185 BC-related genes, including *ERBB2*, and 5 housekeeping genes (*GAPDH*, *PUM1*, *ACTB*, *RPLP0*, and *PSMC4*), employing the nCounter platform (NanoString Technologies). Results showed a significant higher *ERBB2* expression in the HER2-positive BC samples compared with the low expression in the HER2 negative breast samples and the inflammatory tissues samples (¡Error! No se encuentra el origen de la referencia.38).

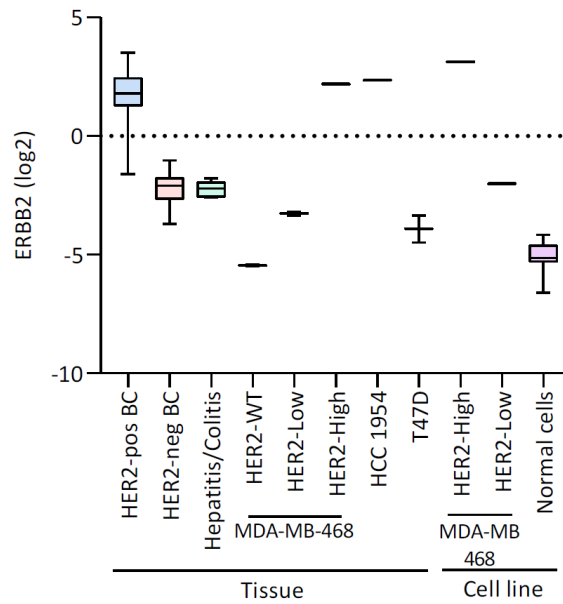


Figure 38. *ERBB2* gene expression in pre-clinical models and tissue samples. *ERBB2* mRNA expression was evaluated using a gene expression panel including BC-related genes using the nCounter platform (NanoString Technologies). *ERBB2* expression was assessed in HER2-positive (n = 132) and HER2-negative (n = 142) BC samples, in inflammatory tissues (hepatitis and colitis; n = 10), in xenografted tumors from NSG mice derived from the following cell lines: HCC1954, T47D, and MDA-MB-468 with wild-type and low HER2 expression (n = 2 per line), and high HER2 expression (n = 1 per line). Additionally, expression was analyzed in MDA-MB-468 cell line with low and high HER2 expression (n = 1 per condition), as well as in normal cell types including keratinocytes, renal cells, osteoblasts, cardiomyocytes, pancreatic cells, ovarian cells and Langerhans cells.

3. Analysis of *ERBB2* mRNA levels in tested preclinical models and the theoretical rationale for the activation threshold of HER2-BBZ

Next, we aimed to correlate *ERBB2* levels with the response observed in functional assays with HER2-BBZ. We have analyzed *ERBB2* levels in both: 1) tissues from tumors xenografted in NSG mice and 2) cell lines with low and high HER2 expression (**Figure 38**).

As expected, we observed high *ERBB2* expression levels in tissues or cell lines positive for HER2 (such as HCC1954 and the MDA-MB-468 HER2-high), as well as in HER2-positive BC patient samples. The tissues or cells exhibiting high *ERBB2* expression are

precisely those where we have consistently observed HER2-BBZ activation, both *in vitro* and *in vivo* (Marzal B thesis, *Receptor antigénico quimérico en linfocitos T para el tratamiento del cancer de mama HER2+*, 2023). These represent the targeted HER2 levels for the HER2-BBZ CAR-T cell product, and therefore, we expect similar results in HER2-positive BC tissues.

Conversely, in tissues with low levels of *ERBB2* mRNA score ($\log_2 < -0.98$), we have observed that these levels are not sufficient to activate HER2-BBz CAR. However, these levels have been sufficient to activate the high-affinity HER2 CAR-T cell product (NIH). This group includes negative controls, such as HER2-negative BC tissue or HER2-negative cell lines (e.g., MDA-MB-468), but also encompasses the MDA-MB-468 HER2-low cell line model and healthy cells. We have further demonstrated the lack of HER2-BBZ activation in these models and thus hypothesize that those *ERBB2* levels are insufficient for HER2-BBZ activation.

4. Soluble HER2 in serum of patients after chemotherapy treatment

During the scientific advice with AEMPS, the Non-clinical area raised a question about the increase in HER2 expression in non-tumor tissues after chemotherapy treatment. A bibliographic review revealed no published data on the induction of HER2 expression by cytotoxic drugs, while existing studies suggest that chemotherapy—alone or combined with anti-HER2 therapies—may significantly reduce HER2 expression in tumor cells, although its potential modulation in healthy tissues remains unexplored due to ethical limitations in obtaining paired biopsies.

We agreed to determine the HER2 protein plasmatic levels on serum of HER2-positive and HER2-negative BC patients, as well as a comparison between expression levels before and after the chemotherapeutic treatment. First, we observed that concentration of HER2 was significantly higher ($p < 0.0001$) in the serum from patients with HER2 positive BC before receiving chemotherapy treatment, compared to HER2 negative BC patients, where HER2 levels were negative (**Figure 39**).

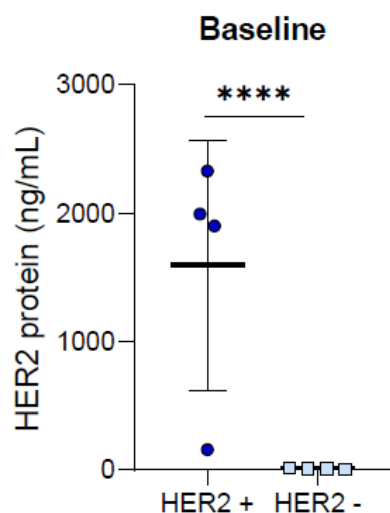


Figure 39. HER2 protein concentration on serum of HER2+ and HER2- metastatic BC patients before initiation of treatment. HER2 concentrations were assessed on serum of cancer patients by an immunoassay using Atellica Solution. Each dot represents one patient.

Moreover, the kinetics of plasmatic levels of HER2 protein were analysed in 4 patients with HER2-positive BC. For that, the basal levels (before taxol-based chemotherapy initiation) and the levels after each treatment cycle were measured (**Figure 40**). In all cases, we observed increased basal levels of plasmatic HER2 protein that were later decreased after the initiation of chemotherapy and tumor burden reduction and maintained negative while the disease was in response.

In the case of patient 4 (**Figure 40 D**), we observed a progressive increase in HER2 levels after the 9th determination, coinciding with tumor progression confirmed by radiology.

After initiating second-line treatment with T-DXd (HER2 monoclonal antibody and cytotoxic compound conjugate), the disease was controlled, and a decrease in plasma HER2 concentration was again observed.

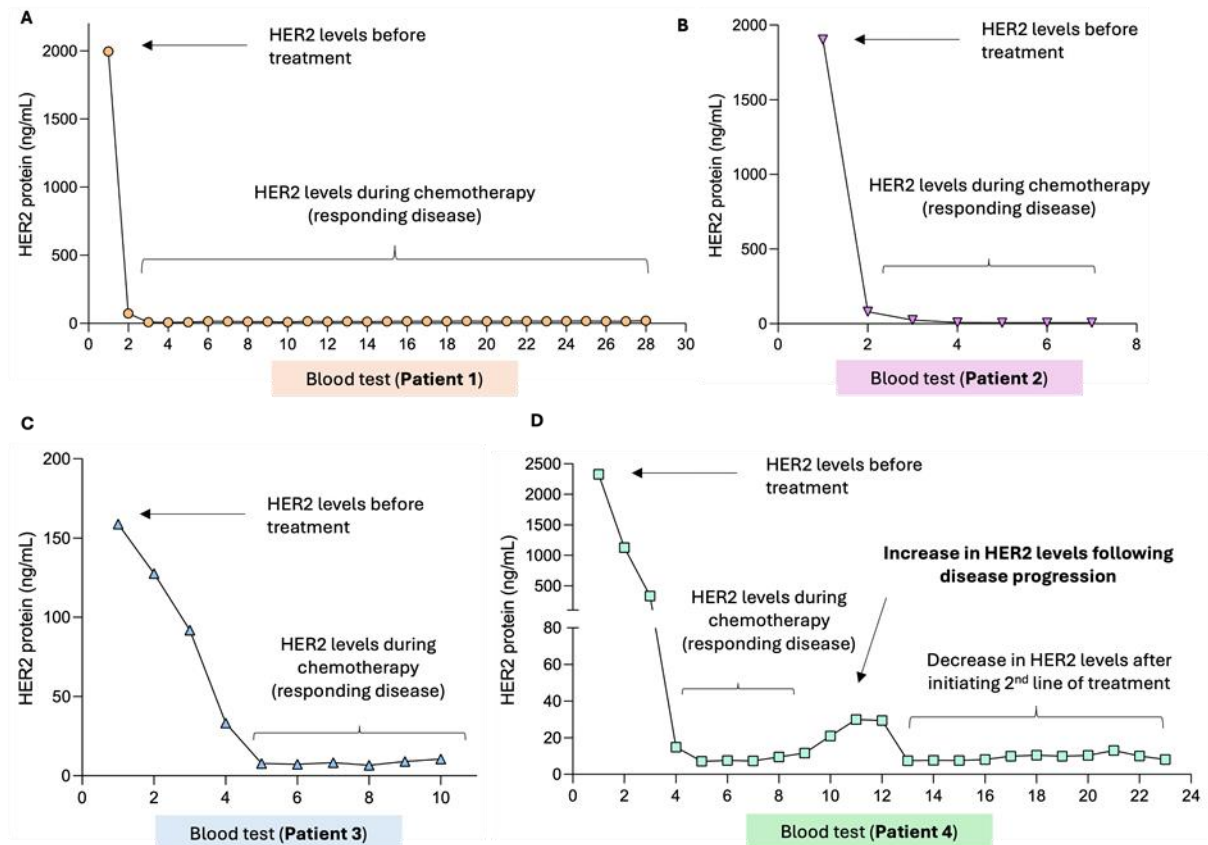


Figure 40. Evolution of soluble HER2 levels in serum from four patients with HER2-positive metastatic BC. A–C) Serum HER2 profiles in patients showing clinical response to taxol-based chemotherapy. D) Serum HER2 trend in a patient experiencing disease relapse. The first data point in each panel corresponds to the baseline measurement obtained before treatment initiation; subsequent values represent HER2 determinations performed throughout the course of disease.

Altogether, these studies show how HER2 concentration is modulated by the HER2-positive tumor load and it is reduced significantly after chemotherapy. The consistent negative levels of HER2 protein during the period when the disease is in response suggest that the HER2 expression on cellular membrane is not increased by the chemotherapeutic effect.

Clinical trial design

Based on previously presented results demonstrating the preclinical efficacy and favorable safety profile of HER2-BBz, the research team progressed their own product into clinical development. This represents a pioneering first-in-human clinical trial in Europe of CAR-T cell therapy targeting HER2 for the treatment of BC. All necessary documentation has been prepared for submission to the Agencia Española del Medicamento y Productos Sanitarios (AEMPS), and the submission was completed in July 2025.

The study, title “A Phase I First-in-Human Dose Escalation Trial to Evaluate the Safety and Efficacy of ARI-HER2 (HER2-BBz CAR-T Cells) in Patients with HER2-Positive Advanced Breast Cancer” is a multicenter study involving Clínic Barcelona Comprehensive Cancer Center, Clínica Universidad de Navarra, and Hospital 12 de Octubre in Madrid. The trial follows a standard 3+3 dose-escalation design to assess the safety and tolerability of ARI-HER2 in patients with HER2-positive metastatic BC who are refractory to all approved therapies.

Key data from the clinical trial are summarized below. For additional information, please refer to the full protocol provided in the Appendix section.

1. Investigational product

ARI-HER2 is an autologous human T-cell product derived from adult peripheral blood T-cells, which are expanded and transduced with a lentiviral vector to express a low-affinity CAR targeting HER2. Participants will undergo lymphoapheresis to collect T-cells from peripheral blood (50–250mL). Before apheresis, a CD3+ T-cell count of at least 100 cells/ μ L and negative serologies are required. Each apheresis product must contain at least 400 million of CD3+ T lymphocytes, or 800 million in the case of cryopreserved apheresis.

ARI-HER2 cells, will be manufactured in a commercial bioreactor (CliniMACS Prodigy, Miltenyi) following a process like that used for other HCB products^{165,166}. The infusion product will be packaged in three 30 mL bags, with the total dose divided into three aliquots, each corresponding to the patient’s cohort and weight. ARI-HER2 cells will be administered intravenously, with fractioned dosing based on our previous experience

with ARI-0001 and ARI0002h cells. We plan to administer between 0.15 and 4.5×10^6 ARIHER2 cells/kg in 3 fractions. Ideally these 3 fractions will be administered within a period of 12 days.

2. Overview of clinical trial design

This study is a first-in-human, multicenter, open-label, prospective, non-randomized, dose escalation clinical trial using a 3+3 design to assess the safety and tolerability of ARI-HER2 in patients with HER2-positive mBC who are refractory to approved treatments. The trial design is summarized in **Figure 41**.

Patients will be enrolled in the study in strict sequential order. The administration of ARI-HER2 cells will be staggered, with each patient being monitored for a minimum of 14 days before the next patient receives treatment.

The study will consist of five distinct phases:

- **Patient Screening:** After obtaining informed consent, a fresh tumor biopsy will be performed to confirm HER2 status. If the results meet the selection criteria, as validated by the medical monitor, a leukapheresis procedure will be conducted to collect sufficient peripheral mononuclear cells.
- **Preparation of the Investigational Drug:** ARI-HER2 cells will undergo a manufacturing process lasting 7–10 days, followed by a two-week period for quality control evaluation.
- **Patient Inclusion:** Once successful production of the investigational product is confirmed, the inclusion and exclusion criteria will be reviewed during the screening visit. Upon approval by the medical monitor, the patient will be officially included in the study.
- **Treatment Period:** This phase will involve lymphodepleting chemotherapy, followed by a fractionated infusion of ARI-HER2 cells.
- **Follow-up:** Patients will be monitored for at least 24 months following the first ARI-HER2 infusion. Additionally, in accordance with good clinical practices for advanced therapies, long-term safety follow-up will be conducted for up to 15 years post-infusion.

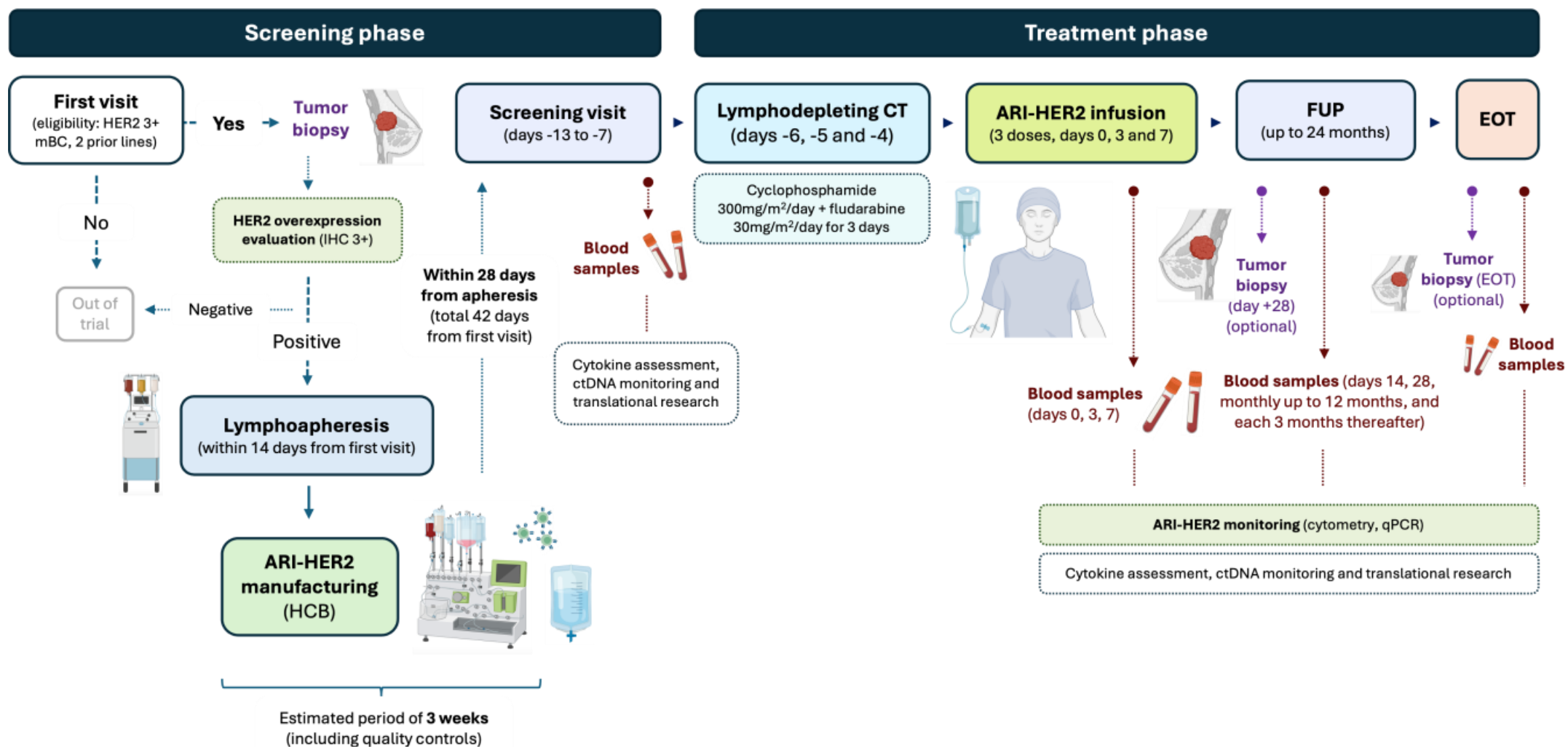


Figure 41. ARI-HER2 study design

3. Dose justification

To the best of our knowledge, there is only one published clinical trial and two clinical cases with CAR-T against HER2. In order to select the dose escalation, the research team has reviewed the previously published phase 1 clinical trials with CAR-T therapy in solid tumors (**Table 5**). These trials show a variable dose range of 1×10^6 - 1×10^8 cells/ m^2 , 1×10^5 - 1×10^8 cells/kg or 2.5×10^8 - 1×10^{10} total cells. Even so, no severe adverse events were observed except for the first trial published in 2010 with a CAR against HER2 that administered 10^{10} total cells and caused the death of one patient. Recently, Qi C et al.^{167,168} have shown that a CAR-T against Claudin18.2 is effective in patients with previously treated metastatic digestive cancer. The dose used in the expansion phase was 2.5×10^8 total cells. Good tolerance and an ORR of 38.8% were observed and a PFS and OS were 4.4 months (95% CI: 3.7, 6.6) and 8.8 months (95% CI: 7.1, 10.2), respectively.

Based on the dose, efficacy and adverse events of the clinical trials cited above, the dose escalation selected for our clinical trial is 1.5 to 4.5×10^6 CAR-T cells/kg intravenously.

Target	Co-stimulation domain	Dose	Tumor Type	Adverse events	Reference
Anti-HER2 (n=1)	CD28 and 4-1BB	10^{10} cells	Colorectal cancer	Death	<i>Morgan et al. Mol Ther 2010</i> ¹²⁸
Anti-HER2 (n=1)	CD28	1×10^8 cells/ $m^2 \times 2$ cycles	Rhabdomyosarcoma	CRS G1	<i>Hegde et al., Nat Commun 2020</i> ¹¹⁷
Anti-HER2 (n=17)	CD28	1×10^6 - 1×10^8 cells/ m^2	Glioblastoma	Seizures and headache G2	<i>Ahmed N et al., JAMA Oncol 2017</i> ¹⁴⁰
Anti-mesothelin (n=27)	CD28	3×10^5 - 6×10^7 CAR T cells/Kg	Mesothelioma, NSCLC, BC	CRS G<2	<i>Adusumilli PS et al., Cancer Discov 2021</i> ¹³⁵
Anti-mesothelin (n=15)	CD28	0.18 - 11×10^6 CAR T cells/Kg	Solid tumors (MSLN+)	Ascites and pericardial/pleural	<i>Wang Z et al., Cell Mol Immunol</i>

				effusion G1-G3	2021 ¹⁶⁹
Anti-claudin 18.2 (n=98)	CD28	2.5x10 ⁸ – 5x10 ⁸ cells	Gastro-esophageal / pancreatic cancer	CRS G1-G2	<i>Qi C et al. Nat Med, 2024</i> ¹⁶⁸
Anti-CEA (n=10)	CD28	1x10 ⁵ - 1x10 ⁸ cells/Kg	Colorectal cancer	None	<i>Zhang C et al, Molecular therapy 2017</i> ¹⁷⁰
Anti-EGFR (n=11)	4-1BB	0,04x10 ⁷ – 2,54x10 ⁷ cells/Kg	NSCLC	Hyperlipase mia G3-4, rash G1-2, dyspnea G1-2	<i>Feng K et al, sci chi 2016</i> ¹⁷¹
Anti-PSMA (n=13)	CD28	1x10 ⁷ - 3x10 ⁸ cells/	mCRPC	CRS G3-G4 Death (1 patient)	<i>Narayan V et al, Nat Med 2022</i> ¹³¹
Anti-claudin 6 (n=22)	4-1BB	1x10 ⁷ - 1x10 ⁸ cells/Kg	Solid tumors (CLDN6+)	CRS G3-G4	<i>Mackensen et al, Nat Med 2023</i> ¹³⁰
Anti-cMET (n=7)	CD28	1x10 ⁸ cells	Melanoma, TNBC	CRS G1-G2	<i>Shah P et al, Cancer Res Commun 2023</i> ¹⁷²
Anti-GD2 (n=4)	4-1BB	1x10 ⁶ cells	Diffuse midline gliomas	CRS G1-G2	<i>Majzner R et al, Nature 2022</i> ¹³³
Anti-GD2 (n=27)	CD28	3-10x10 ⁶ /kg cells	Neuroblastoma	CRS G1-G2	<i>Del Bufalo F et al, NEJM 2023</i> ¹³²
Anti-EGFR/IL13R (n=6)	CD28	1-2.5x10 ⁷ cells	Glioblastoma	ICANS G3 CRS G1-G2	<i>Bagley S et al, Nat Med 2024</i> ¹⁷³
Anti-IL13R (n=65)	4-1BB	10-200x10 ⁶ cells	High grade glioma	ICANS G3	<i>Brown C et al, Nat Med 2024</i> ¹⁷⁴

Table 5. Published phase I trials with CAR-T therapies for patients with solid tumors. CRS: cytokine-release syndrome; ICANS: immune effector cell-associated neurotoxicity syndrome; NSCLC: non-small cell lung cancer; mCRPC: metastatic castration-resistant prostate cancer; TNBC: triple-negative BC; GPC3: glypican 3.

To minimize the risk of on-target off-tumor toxicity and cytokine release syndrome, the dose will be fractionated and administered in 3 doses over 7 days: 10% of the total on day 0, % on day +3 and 60% on day +7.

Additionally, as a risk minimization measure and to evaluate potential toxicities, the study will follow a dose-escalation 3+3 design, subdivided into 3 cohorts.

Three cohorts have been defined in **Table 6**:

- The **first cohort** would receive between $0.15\text{--}1.5 \times 10^6$ ARI-HER2 cells/Kg in three fractions corresponding to 10, 30 and 60% respectively (0.15 , 0.45 and 0.90×10^6 ARI-HER2 cell/Kg).
- The **second cohort** would receive between $0.30\text{--}3 \times 10^6$ ARI-HER2 cells/Kg in three fractions corresponding to 10, 30 and 60% respectively (0.30 , 0.90 and 1.8×10^6 ARI-HER2 cell/Kg).
- The **third cohort** would receive between $0.45\text{--}4.5 \times 10^6$ ARI-HER2 cells/Kg in three fractions corresponding to 10, 30 and 60% respectively (0.45 , 1.35 and 2.7×10^6 ARI-HER2 cell/Kg).

A minimum follow-up of 14 days will be required after the administration of the last dose fraction of each patient before treating the next patient.

Cohort	Total dose	Fraction 1; 10% (aliquot)	Fraction 2; 30% (aliquot)	Fraction 3; 60% (aliquot)
1	1.5×10^6	0.15×10^6 (0.15)	0.45×10^6 (0.45)	0.9×10^6 (0.9)
2	3×10^6	0.3×10^6 ($0.15+0.15$)	0.9×10^6 (0.9)	1.8×10^6 (1.8)
3	4.5×10^6	0.45×10^6 (0.45)	1.35×10^6 ($0.9+0.45$)	2.7×10^6 ($0.9+1.8$)

Table 6. Dose escalation for ARI-HER2

4. Trial objectives

4.1 Co-primary objectives and endpoints

The co-primary objectives of the clinical trial are to:

- Assess the **safety and tolerability** profile of ARI-HER2.
- Determine the **recommended phase 2 dose** for the expansion phase (RP2D).

The primary endpoints of this study are:

- **Incidence of grade 3-5 adverse events** (AEs) assessed by NCI Common Terminology for Classification of Adverse Events (CTCAE) version 5.0 during

the treatment period.

- Any grade of AE assessed by CTCAE possibly related to treatment (lymphodepletion chemotherapy and/or HER2CAR) leading to therapy discontinuation.
- **Dose-limiting toxicity (DLT)** defined as the presence of grade 3-5 AEs related to ARI-HER2 assessed by CTCAE v5.0 occurring within 21 days immediately following the last HER2-CAR infusion.
- **Maximum tolerated dose (MTD)** defined as the highest dose of HER2-CAR that does not cause grade 3-5 AEs (based on CTCAE v5.0) identified as DLT.

4.2 Secondary objectives and endpoints

The secondary objectives of the clinical trial are to:

- Evaluate the **efficacy** of ARI-HER2 in patients with R/R HER-2 positive metastatic BC.
- Assess the treatment **compliance** of ARI-HER2.
- Evaluate the persistence of ARI-HER2 in blood.
- Evaluate the incidence, severity and duration of **cytokine release syndrome** and **neurotoxicity syndrome** in subjects after ARI-HER2 administration.
- Assess **middle** and **long-term toxicity**.
- Evaluate the **association of the HER2DX genomic** test with clinical outcomes.

The secondary endpoints of this study are:

- **Clinical Benefit Rate (CBR)** defined as the percentage of patients who have a complete response (CR), partial response (PR) or stable disease (SD) lasting >24 weeks, based on local investigator's assessment according to iRECIST criteria v1.1 (see section 8.1 for additional details).
- **Objective response rate (ORR):** locally determined as the best response (defined as CR and PR) by the investigator according to RECIST 1.1 criteria after treatment administration (see section 8.1 for additional details).

- **Duration of response (DOR)** defined as the time from the first occurrence of a documented objective response to disease progression as determined locally by the investigator using RECIST v1.1 or death (whichever occurs first). See section 8.1 for additional details.
- **Progression-free survival (PFS)** defined as the time from administration of the ARI-HER2 product to disease progression or death (whichever is earlier). See section 8.1 for additional details.
- **Overall survival (OS)** defined as the time from administration of the ARI-HER2 product to death from any cause (see section 8.1 for additional details).
- All **changes in treatment administration** including delays, interruptions, reductions or discontinuation and the main reason(s) for these changes.
- CAR persistence defined as **percentage of HER2CAR** in the blood weekly during the first month, monthly until the first year and every three months until 2 years of follow-up.
- **Cytokine induction** defined as the type and number of cytokines released into the blood following HER2CAR infusion, as well as at which time point it occurs.
- **Incidence and grade** of cytokine release syndrome (**CRS**) and neurotoxicity syndrome (**ICANS**) from ARI-HER2 infusion and during follow-up.
- **Incidence of grade 3-5 adverse events** (AEs) assessed by NCI CTCAE version 5.0 at months 3, 6 and 12 after ARI- HER2 infusion.
- **Correlation** between the expression of gene signatures with validated prognostic value in HER2-positive BC assessed by **HER2DX genomic test** with **efficacy** and **survival** outcomes (CBR, ORR, DOR, PFS, OS).

4.3 Exploratory objective

The exploratory aim of this study is to evaluate translational datasets derived from tumor biopsies and peripheral blood samples collected during the clinical trial. These biospecimens will enable a series of subsequent investigations, including immunophenotyping, immunohistochemistry, RNA sequencing, gene expression

profiling and whole-genome DNA sequencing. All samples will be cryopreserved in a biobank under stringent conditions until such analyses are initiated.

5. Inclusion and exclusion criteria

Inclusion criteria

Subjects are eligible for the study if all the following apply:

1. Able to understand the trial and willingly sign the informed consent.
2. Is **≥18 years** of age at the time of signing the informed consent.
3. Eastern Cooperative Oncology Group (**ECOG**) performance status of **0 or 1**.
4. Histological diagnosis of **HER2-positive metastatic or unresectable BC** according to American Society of Clinical Oncology guidelines.
5. Locally confirmed HER2+ status with **IHC score 3+** in a newly obtained tumor sample (mandatory pre-treatment biopsy for local confirmation). HER2-positive tumors with IHC 2+ and amplified ISH will not be considered for inclusion.
6. Prior disease progression or intolerance to standard HER2-targeted therapies:
 - a) Patients must have received **at least 2 lines of HER2-directed therapy** for their metastatic disease, **including trastuzumab-deruxtecan (T-DXd)**. Prior HER2-targeted neo-adjuvant or adjuvant therapy that resulted in relapse within 12 months of the completion of therapy will be considered a line of treatment for metastatic disease.
 - b) Based on the physician's choice, participants' eligibility, and institutional and local guidelines, participants may also have received post-T-DXd therapy, for example, a tucatinib-based regimen and/or T-DM1.
7. At least **one** measurable and/or **evaluable lesion** according to RECIST 1.1 criteria.
8. Life expectancy of at least 3 months, in the opinion of the investigator.
9. **Adequate organ function** determined within 28 days prior to enrolment, defined as follows:
 - Hematological (without need for transfusion and or growth factor support within 15 days):

- Absolute neutrophil count $\geq 1.5 \times 10^9/L$
 - Platelet count $\geq 100,000/mm^3$; participants with stable platelet count from 75,000 to 100,000/ mm^3 may be included with approval from the medical monitor.
 - Hemoglobin ≥ 9 g/dL
 - Lymphocytes $> 0.1 \times 10^9/L$
 - Renal
 - Serum creatinine $\leq 1.5 \times$ ULN or 24-hour creatinine clearance ≥ 50 mL/min (Cockcroft - Gault) for subjects with creatinine levels $> 1.5 \times$ ULN.
 - Hepatic
 - Serum bilirubin $\leq 1.5 \times$ ULN OR direct bilirubin $\leq$ ULN for a subject with total bilirubin level $> 1.5 \times$ ULN, except for patients with a diagnosis of Gilbert's syndrome.
 - AST and ALT $\leq 2.5 \times$ ULN OR $\leq 5 \times$ ULN for subjects with liver metastases.
 - Coagulation: International normalization ratio (INR) or prothrombin time (PT) $\leq 1.5 \times$ ULN.
 - Lung function: CTCAE v5.0 grade ≤ 1 dyspnea and SatO2 $\geq 92\%$.
 - Cardiac: left ventricular ejection fraction (LVEF) $\geq 50\%$ as determined by either echocardiogram or MUGA obtained within 12 weeks before the first dose of study intervention.
10. Last dose of prior anti-cancer therapy at least 28 days or 5 half-lives prior to ARI-HER2 infusion.
11. Participants with history of **stable CNS metastases are eligible**, provided the following criteria are met:
- a) Treated brain metastases, in response to local or systemic treatment. If steroid therapy is required for the management of neurologic symptoms, the maximum allowed dose will be prednisone ≤ 10 mg/day or equivalent.
 - b) Radiological stability of CNS metastases on MRI at the time of screening.
 - c) If new brain metastases are diagnosed during screening, the patient may

receive local treatment with radiotherapy and undergo an MRI scan 4 weeks after completion. If stability is confirmed, the patient may then proceed to the treatment phase.

12. Adequate venous peripheral access for apheresis.
13. Ability to provide a newly obtained tumor biopsy (mandatory for screening). If subjects have only one or no accessible lesion, their inclusion will be discussed with the research team. Additional biopsies for translational research will be offered to the patients at 1st reevaluation (D28) and progression (EOT).
14. Patients must agree not to participate in another interventional study while receiving study drug (subjects who are currently in the Follow-up Period of an interventional clinical trial are allowed).

Exclusion criteria

Subject will be excluded from participation if any of the following apply:

1. Known sensitivity to any of the products or components to be administered. Contraindication to receive lymphodepleting chemotherapy.
2. Known or suspected **leptomeningeal disease or spinal cord compression**.
3. Toxic effects of prior therapies not resolved to CTCAE v5.0 grade 1 or less, with the following exceptions: alopecia, neuropathy resolved to $\leq$ Grade 2, fatigue $\leq$ Grade 2, or endocrinopathy $\leq$ Grade 2.
4. Incompatible concurrent medication: systemic therapeutic **corticosteroids** must be discontinued 72 hours before CAR-T infusion. However, patients who require replacement therapy for adrenal insufficiency may be enrolled if steroid treatment doses do not exceed 10 mg of prednisone or equivalent.
5. History or evidence of symptomatic autoimmune pneumonitis, glomerulonephritis, vasculitis, or other symptomatic autoimmune disease, or active autoimmune disease or syndrome that has required systemic treatment in the past 2 years (e.g., disease-modifying agents, corticosteroids, or immunosuppressive drugs), except for vitiligo or resolved childhood asthma/atopy. Replacement therapy (e.g., thyroxine, insulin, or physiologic corticosteroid replacement therapy for adrenal or pituitary insufficiency) is not considered

systemic treatment.

6. Uncontrolled or significant **cardiovascular disease** including any of the following:
 - a. History of myocardial infarction or unstable angina within 6 months prior to enrolment.
 - b. History of symptomatic congestive heart failure (New York Heart Association Class II to IV)
 - c. Ventricular arrhythmia requiring therapy
7. Previous history of allogeneic hematopoietic cell or solid organ **transplantation**.
8. **Active infection** within 14 days prior to study treatment.
9. Known history of human immunodeficiency virus (**HIV**) infection.
10. History of active tuberculosis.
11. Positive Hepatitis B virus antibodies and surface antigen (indicating acute HBV or chronic HBV) or Hepatitis C (HCV RNA (qualitative)). Hepatitis C RNA testing is not required in subjects with negative Hepatitis C antibody testing. Hepatitis B virus antibodies are not required in subjects with negative Hepatitis B surface antigen.
12. History of **other malignancy** within the past 2 years with the following exceptions at the time of enrollment: adequately treated non-melanoma skin cancer without evidence of disease, adequately treated cervical carcinoma in situ without evidence of disease, adequately treated breast ductal carcinoma in situ without evidence of disease, prostatic intraepithelial neoplasia without evidence of prostate cancer or adequately treated superficial or in-situ carcinoma of the bladder without evidence of disease.
13. Live vaccine against infectious diseases administered within 28 days of study treatment.
14. Unlikely to comply with all protocol-required study visits or procedures, to the best of the subject and investigator's knowledge.
15. History or current evidence of any condition, therapy, or laboratory abnormality that might confound the results of the trial, interfere with the subject's participation for the full duration of the trial, or is not in the best interest of the subject to participate, in the opinion of the treating investigator.
16. If women or men, refusal to the use of approved contraceptive methods (e.g. barrier device, intrauterine device, abstinence) during the study and for at least 12

months following the last dose of the study cell infusion and until no CAR-T cells can be detected after two consecutive PCR tests.

17. Pregnant or breast-feeding women.

6. General adverse events management

Once acceptance criteria are met, and the patient has completed lymphodepleting chemotherapy, the first fraction of ARI-HER2 cell will be delivered to the patient's room and thawed in situ in a water bath heated at 36-38°C. The same procedure will be repeated for second and third fraction provided there aren't signs or symptoms of CRS/ICANS requiring specific medical intervention.

The administration of the **second and/or third fraction will be avoided in case of** any of the following situations after the previous dose (grades are defined according to CTCAE 5.0 criteria).

- **Grade ≥ 3 CRS or ≥ 2 ICANS (Table 7).** In the case of grade 2 CRS or grade 1 ICANS, the administration of further ARI-HER2 fractions should be discussed with the medical monitor.
- Clinical and/or microbiological evidence of an **active infection**.
- Potentially **severe on-target off-tumor toxicity**, defined as: grade ≥ 3 diarrhea, grade ≥ 3 elevation of liver enzymes (ALT, AST), grade ≥ 2 dyspnea or hypoxia, especially in the presence of radiological signs of pneumonitis and/or echocardiographic confirmation of ejection fraction decrease, and/or any other grade ≥ 3 toxicity at investigator criteria. If tumor lysis syndrome is suspected, hydration and other conventional measures (e.g. allopurinol 300 mg/24 h for a duration ranging from 7 days to one month depending on the presence/absence of cell expansion) may be used at medical discretion.

Toxicity grade	Medication and global management	ARI-HER2 management
Cytokine release syndrome (CRS)		
Grade 1 (fever [≥37.8°C] without hypotension or hypoxia)	Consider tocilizumab 8mg/kg (max 800 mg) if persistent fever (>24h). Tocilizumab can be repeated after 24h. If no response after 2 doses, consider dexamethasone (DXM) IV 10mg/6h. Supportive therapy (fluids, antipyretics). Close monitoring in an intermediate care ward.	Continue with fractions of ARI-HER2 after 36- 48h without fever.
Grade 2 (fever + hypotension not requiring vasopressors and/or hypoxia responding to low-flow oxygen)	Tocilizumab 8mg/kg (max 800 mg) + DXM IV 10mg (repeat both drugs if no improvement after 8 h) + supportive therapy if not initiated. If no response after first 2 doses, continue DXM IV 10mg/6h. Alert local intensive care unit (ICU).	Continuing ARI-HER2 should be discussed with the medical monitor (MM).
Grade 3 (fever + hypotension requiring 1 pressor and/or hypoxia requiring high-flow oxygen)	Manage in ICU. If no response to 2 doses of tocilizumab 8mg/kg + DXM IV 10mg/6h, consider anakinra 8mg/kg each 24h. If no response to anakinra, consider single dose of siltuximab IV 11 mg/kg. Individualize further intensive care.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 4 (fever + hypotension requiring >1 pressor and/or hypoxia requiring ventilation/intubation/positive pressure)		
Immune-effector cell-associated neurotoxicity syndrome (ICANS)		
Grade 1 (ICE* 7-9, mild encephalopathy, awakens spontaneously; no seizures, no motor findings, no cerebral edema).	Start DXM IV 10mg/6h. Perform EEG, lumbar puncture and neuroimaging (MRI); rule out CNS infection. Monitor closely (ICE every 6-8 hours). Individualize supportive care (oral intake, raised head at 30°C).	Continuing ARI- HER2 must be discussed with MM.
Grade 2 (ICE* 3-6, awakens to voice; no seizures, no motor findings, no cerebral edema).	Transfer to ICU. If no improvement after 24-48 h with DXM IV 10mg/6h, escalate to DXM IV 20mg/6h. If no response after 24-48 h, start anakinra (8mg/kg each 24h).	Avoid further fractions of ARI-HER2. Alert MM.
Grade 3 (ICE* 0-2, awakens to tactile stimulus; any clinical seizure or epileptic activity on EEG that resolves with intervention).		
Grade 4 (stupor/coma, unable to perform ICE*; prolonged [>5 min] or repetitive clinical or electrical seizures;focal motor weakness; diffuse cerebral edema or papilledema, decerebrate/decorticate posturing, cranial nerve VI palsy, Cushing triad).	If no response after 24h, consider single dose of siltuximab IV 11 mg/kg. Individualize further neurointensive treatment. In refractory cases, consider dasatinib 140mg/24h to neutralize CAR activity and/or other individualized measures (plasmapheresis.	

	intrathecal treatment).	
Immune-effector cell-associated hemophagocytic-like syndrome (IEC-HS)		
Grade 2 (not severe clinical manifestations).	Anakinra 8mg/kg each 24h. Start DXM IV 10mg/6h if not initiated. Consider transfer to ICU.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 3 (severe, coagulopathy with bleeding requiring transfusion, acute kidney injury, hypotension, respiratory distress).	If no response, consider single dose of siltuximab IV 11 mg/kg. In refractory cases, consider alternative agents (ruxolitinib, low-dose etoposide).	
Grade 4 (intubation, dialysis, intensive intervention required, life-threatening consequences).		
On-target off-tumor toxicity (diarrhea, pneumonitis, altered liver enzymes, ejection fraction decrease, any other AEs related to damage of HER2-expressing tissues), and other AEs related to ARI-HER2 (<u>NOT</u> including those related to lymphodepleting chemotherapy)		
Grade 1	Specific supportive therapy.	Consider interrupting ARI-HER2 in potentially severe AEs (pneumonitis, ejection fraction decrease) or no improvement within 48-72 hours.Consult with MM.
Grade 2		
Grade 3	Consider steroids and/or other immunosuppressive drugs. In severe and/or refractory cases, transfer to ICU and consider dasatinib 140mg/24h and/or high- dose cyclophosphamide.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 4		
AEs related to lymphodepleting chemotherapy		
Neutropenia		
Grade 1 (>1500/mm ³).	Not indicated.	Do NOT suspend ARI-HER2 fractions.
Grade 2 (1000-1500/mm ³).		
Grade 3 (500-1000/mm ³).		
Grade 4 (<500/mm ³).	Initiate G-CSF (Filgrastim 300-480 ug/d) + prophylactic levofloxacin 500mg/d and fluconazole 400mg/d until neutrophils >500-1000/mm ³ . Consider escalating antibiotics if fever. Intensify anti-fungal prophylaxis to posaconazole or isavuconazole for patients receiving high doses of DXM and/or other	

	immunosuppressants.	
Anemia		
Grade 1 (>10g/dL).	Not indicated.	Do NOT suspend ARI-HER2 fractions.
Grade 2 (8-10g/dL).		
Grade 3 (<8g/dL).	Blood transfusion if Hb <7g/dL. Individualize for Hb 7-8 g/dL. Irradiated products should be used for transfusion. Consider erythropoietin in refractory/persistent cases.	
Grade 4 (life-threatening).	Blood transfusion and support therapy. Irradiated products should be used for transfusion.	Discuss with MM.
Thrombopenia		
Grade 1 (>75000/mm ³).	Not indicated. Consider suspending prophylactic anticoagulation if initiated.	Do NOT suspend ARI-HER2 fractions.
Grade 2 (50000-75000/mm ³).		
Grade 3 (25000-50000/mm ³).		
Grade 4 (<25000/mm ³).	Consider platelet transfusion if <20000/mm ³ or active bleeding. Irradiated products should be used for transfusion. Consider eltrombopag 100- 150mg/24h in refractory/persistent cases.	
Lymphopenia		
Grade 1 (>800/mm ³).	Not indicated.	Do NOT suspend ARI-HER2 fractions.
Grade 2 (500-800/mm ³).		
Grade 3 (200-500/mm ³).	Initiate prophylaxis with acyclovir 400mg/12h vo + cotrimoxazole 800mg/24h at least for 6-12 months	
Grade 4 (<200/mm ³).	and until CD4 count >200/mm ³ .	

Table 7. AEs management. The recommended management of potential AEs according to the toxicity grade.

***ICE score for neurological toxicity assessment:** Orientation (year, month, city, hospital) 4 points, Naming (ability to name three objects): 3 points, Following commands (ability to follow simple commands) 1 point, Writing (ability to write a standard sentence) 1 point, Attention (ability to count backwards from 100 to 0 by 10) 1 point.

DISCUSSION

STUDY 1: IGG PROJECT

The TME has emerged as a critical determinant of tumor behavior and therapeutic response in breast cancer. Among its components, TILs have been extensively studied as prognostic and predictive biomarkers, as well as for their role in mediating resistance through T-cell dysfunction¹⁷⁵. While TIL assessment remains a useful clinical tool, its utility is limited by interobserver variability and incomplete capture of immune complexity.

Our work builds on this by focusing on the IGG signature, a 14-gene expression profile incorporated into HER2DX¹⁹, the first genomic assay developed for HER2-positive breast cancer. The IGG signature has consistently shown strong and independent prognostic value beyond TILs, but its biological underpinnings and broader implications have remained poorly understood^{19,62–64}. By integrating spatial proteomics, single-cell transcriptomics, and immune receptor repertoire sequencing, we provide a multidimensional characterization of the IGG signature. In particular, our findings highlight its role as a marker of immune activation, TLS biology, clonal diversity, and even systemic immune fitness associated with long-term survival.

IGG and the immune-inflamed TME

We found that IGG-high tumors exhibit a prototypically “immune-inflamed” microenvironment, with coordinated enrichment of plasma cell (e.g., CD27), T-cell (CD3), and antigen-presenting cell (APC) markers (CD40, IDO1), together with reduced expression of stromal-remodeling and immunosuppressive proteins such as fibronectin and CD163. This suggests that the IGG signature is not merely a proxy for B-cell infiltration, but rather a systems-level marker of adaptive immune coordination. IGG-high tumors appear to represent a “networked” immune state, linking B-cell maturation and plasma-cell activity with T-cell engagement and APC-driven antigen presentation.

Mechanistically, several points are noteworthy. CD27 and CD40 indicate active germinal center–like biology, supporting affinity maturation and durable immune memory. Although IDO1 is often described as immunosuppressive, its induction here

likely reflects interferon-rich inflammatory signaling. Meanwhile, reduced CD163 and fibronectin suggest attenuation of macrophage-driven immunoregulation and extracellular matrix stiffness, creating a more permissive microenvironment for immune infiltration. Together, these observations point toward an active, inflamed, and less fibrotic TME.

These findings integrate well with our spatial proteomics, where immune-inflamed signals were strongest in CD45+ stromal regions, and were amplified in TLSs. They are also consistent with our clonotype data, which demonstrated broader BCR and TCR polyclonality in IGG-high tumors. Importantly, our results parallel observations from large correlative analysis from clinical trials such as CALGB 40601¹⁷⁶, where B-cell immune-related gene signatures correlated with higher pCR and better survival, while stromal signatures predicted poorer outcomes. Our work extends those observations by adding spatial and repertoire resolution, showing that IGG not only tracks with therapeutic response but also captures the architecture and diversity of the underlying immune reaction.

From a translational perspective, an IGG-high, APC-rich, and less fibrotic TME may enhance the efficacy of HER2-targeted antibodies such as trastuzumab and pertuzumab, which rely partly on Fc-mediated effector functions. By contrast, IGG-low tumors enriched for fibronectin and CD163 may represent less permissive microenvironments, suggesting opportunities for stromal or myeloid reprogramming strategies to improve immune accessibility.

TLSs and B-cell biology

Our spatial analysis revealed that IGG expression strongly associates with the presence of TLSs, which provide an organized niche for B-cell activation, plasma-cell differentiation, and antigen presentation. TLSs are ectopic lymphoid aggregates that resemble secondary lymphoid organs, containing B-cell follicles, germinal center-like structures, and T-cell zones supported by dendritic cells. Within the TME, TLSs act as local immune training grounds: they facilitate affinity maturation, support the generation of plasma cells and memory B cells, and sustain T-cell priming through

continuous antigen presentation^{85,177,178}. This microanatomical organization allows for adaptive immune responses to occur in situ, potentially bypassing the need for trafficking to lymph nodes.

In breast cancer, TLSs have historically been identified through histopathological assessment, but this method is inherently limited by interobserver variability and the two-dimensional nature of tissue sections¹⁷⁹. Moreover, TLSs are often heterogeneous in their composition and degree of maturation, complicating their classification¹⁸⁰. By contrast, the IGG signature offers a scalable molecular surrogate that captures TLS-like biology through bulk transcriptomics. Our data show that IGG-high tumors, even in the absence of histologically detectable TLSs, display protein and transcriptomic features consistent with TLS-like immune activity, suggesting that IGG reflects not only visible structures but also the functional biology of organized B- and T-cell interactions within the stroma.

The clinical implications of TLSs are substantial. Across multiple solid tumors, the presence and density of TLSs have been associated with improved prognosis and, more recently, with enhanced responses to immune checkpoint inhibitors^{181–184}. TLSs are thought to promote “immune-inflamed” phenotypes characterized by ongoing antigen presentation and sustained cytotoxic T-cell responses, making them a compelling biomarker for immunotherapy benefit. In breast cancer, their role has been less well defined, in part because of challenges in reproducible detection. The ability of IGG to capture TLS-like activity offers a path forward by providing a standardized and clinically applicable molecular readout.

Mechanistically, the link between IGG and TLS biology underscores the contribution of B cells to shaping the TME. B cells are not passive players confined to antibody production; rather, they orchestrate adaptive responses by supporting T-cell activation, producing cytokines, and acting as antigen-presenting cells. Within TLSs, activated B cells and plasma cells generate diverse antibody repertoires, which can enhance tumor cell killing directly through complement and Fc-mediated effector mechanisms or indirectly through antigen spreading and T-cell recruitment. Our

findings of increased plasma cell and APC markers in IGG-high tumors support this integrated model.

From a translational perspective, TLSs—and by extension, IGG—could serve multiple roles. First, they may stratify patients more likely to benefit from HER2-targeted therapies and novel immunotherapy combinations. Second, TLS biology could be harnessed therapeutically: preclinical work has shown that TLS induction can be promoted by cytokines such as lymphotoxin or by therapeutic cancer vaccines, raising the possibility of actively reprogramming TLS-negative tumors. Third, TLS-associated markers, including IGG, may offer a noninvasive way to monitor immune activation during therapy, providing dynamic readouts of treatment efficacy.

Taken together, our findings suggest that the IGG signature captures the biological essence of TLSs within the HER2-positive TME, offering a reproducible, scalable, and clinically deployable marker of organized antitumor immunity. By bridging histology with molecular readouts, IGG has the potential to move TLSs from a research biomarker to a routine clinical tool in breast cancer and beyond.

Immune clonality and diversity

We also observed that IGG-high tumors exhibit greater polyclonality of both B- and T-cell repertoires, reflecting a more diverse and evenly distributed set of productive rearrangements in the adaptive immune system. Such diversity is generally regarded as a hallmark of effective antitumor immunity. A polyclonal immune repertoire broadens the capacity to recognize a wide range of tumor-associated and neoantigenic epitopes, thereby increasing the probability of mounting an effective response against heterogeneous tumor cell populations^{90,92,93}. In contrast, oligoclonal or highly skewed repertoires may be more vulnerable to immune escape, as tumors can evade immune surveillance by downregulating or mutating the few epitopes being targeted.

The concept that repertoire diversity translates into improved tumor control is consistent with prior evidence. In the CALGB 40601 trial, heterogeneity in BCR repertoire metrics was associated with differences in treatment response, and in NeoALTTO, high expression of immune-related gene signatures—including those linked to adaptive immune activity—was correlated with increased pathological complete response rates^{159,185}. Our results extend these observations by providing direct evidence that IGG-high tumors harbor not only an inflamed phenotype but also a broad and balanced immune receptor repertoire, which together may contribute to their favorable prognosis.

Mechanistically, BCR diversity supports the generation of antibodies targeting multiple tumor antigens, which can mediate tumor clearance through complement activation, direct neutralization, and Fc-mediated effector functions. In parallel, TCR diversity ensures recognition of a broad spectrum of tumor-derived peptides presented on MHC molecules, enabling cytotoxic T-cell responses against multiple tumor subclones. This dual breadth of recognition is likely to limit clonal immune evasion and to sustain long-term immune pressure on the tumor. Moreover, repertoire diversity may facilitate epitope spreading, whereby initial immune responses against a limited set of antigens broaden to include secondary targets, amplifying the antitumor effect over time.

Beyond its prognostic value, immune repertoire diversity has significant translational potential. Clonality metrics could serve as dynamic biomarkers of treatment efficacy: longitudinal monitoring of BCR/TCR repertoires during neoadjuvant or systemic therapy may reveal whether treatment expands pre-existing clonotypes, induces *de novo* clones, or drives oligoclonal dominance associated with resistance^{92,93}. Such insights could help identify patients who are responding immunologically before radiographic or pathological responses are evident.

In addition, dominant clonotypes identified in tumors could be leveraged to develop personalized immunotherapies. For example, tumor-reactive BCR sequences could inform the discovery of novel monoclonal antibodies or guide the engineering of antibody–drug conjugates. Likewise, TCR clonotypes specific for tumor neoantigens could be isolated and expanded for adoptive transfer, or used to design TCR-

engineered therapies targeting intracellular proteins not accessible to conventional antibody drugs. This approach could be particularly valuable for patients with limited treatment options or those with tumors resistant to HER2-targeted therapies.

Finally, our findings raise the intriguing possibility that repertoire diversity, as captured by IGG, may not only predict immediate treatment outcomes but also contribute to long-term survival and immune resilience. By maintaining a broad arsenal of B and T cells capable of responding to evolving tumor antigens, IGG-high patients may be better protected against relapse and may also display enhanced resistance to other diseases, including infections and age-related immune decline.

Taken together, these findings highlight repertoire diversity as a critical dimension in shaping tumor–immune interactions. The ability of the IGG signature to reflect this diversity underscores its potential as both a biomarker of clinical outcome and a guide for developing next-generation immunotherapies.

IGG as a marker of longevity

Perhaps the most striking and novel observation from our study is the association between IGG expression and overall survival in patients who remained recurrence-free—a concept we termed “longevity.” Unlike classical prognostic biomarkers that primarily predict the risk of relapse or progression, IGG appears to capture a broader dimension of immune competence that extends into survivorship. This reframes IGG not only as a tumor-specific biomarker but also as a potential marker of systemic immune fitness.

This relationship was consistent across three large breast cancer cohorts and persisted even after excluding relapsed patients, reducing the possibility that the association was simply a reflection of lower recurrence risk. Moreover, the association extended to multiple other cancer types in the TCGA dataset, suggesting that IGG may serve as a pan-cancer biomarker of immune resilience. This cross-tumor consistency supports the idea that IGG reflects fundamental aspects of adaptive immunity, rather than biology unique to breast cancer.

The concept that immune competence influences long-term survival aligns with a growing body of evidence from immunology and aging research. Studies in centenarians, for example, have shown a marked shift from naïve to memory B cells, indicative of a more robust and antigen-experienced immune system^{186,187}. These individuals often maintain diverse and functionally competent B-cell repertoires, which likely contribute to enhanced resilience against infections, chronic inflammatory conditions, and malignancies. Our finding that IGG-high tumors are associated with both polyclonal immune repertoires and improved longevity provides a direct bridge between tumor immunology and the biology of healthy aging.

Mechanistically, IGG-high expression may reflect the presence of long-lived plasma cells and memory B cells capable of sustaining antibody-mediated immune surveillance over extended periods. This is particularly relevant in the context of breast cancer survivors, who may live decades beyond their initial diagnosis. In these individuals, a robust immune memory could act as a “protective buffer,” reducing vulnerability to late recurrences or even unrelated life-threatening diseases. The association of IGG with T-cell clonality and diversity further strengthens the case that IGG reflects a broad and durable adaptive immune landscape.

From a clinical perspective, the identification of IGG as a marker of longevity has important implications. First, it suggests that prognostic assessment in early breast cancer could be broadened beyond recurrence risk to include metrics of long-term survival and survivorship quality. Second, IGG could be explored as a stratification tool in survivorship programs, identifying individuals who may benefit from closer monitoring, preventive interventions, or immunizations. Third, IGG may inform therapeutic strategies aimed at promoting immune health in cancer survivors—for instance, by guiding interventions that preserve or enhance B-cell and T-cell diversity following cytotoxic or targeted therapies.

More broadly, IGG may help shift the paradigm in oncology from a narrow focus on short-term outcomes such as recurrence and pCR to a more holistic view of patient trajectories that includes immune resilience, survivorship, and healthy aging. By linking

tumor biology to systemic immune fitness, IGG highlights the possibility that cancer and aging research can inform each other, opening new avenues for improving both cancer-specific outcomes and overall life expectancy.

Molecular underpinnings: CIGGs

Our analysis identified seven “core IGG genes” (CIGGs)—TNFRSF17 (BCMA), IL2RG, POU2AF1, CD27, IGJ (JCHAIN), CD79A, and PIM2—that collectively drive the association with longevity. These genes represent functionally distinct yet convergent elements of adaptive immunity, linking B-cell maturation, plasma-cell survival, immunoglobulin production, and T-cell co-stimulation. Together, they form an interconnected network that underlies the biology captured by the IGG signature.

Each CIGG contributes unique functional roles. TNFRSF17 (BCMA) is a key receptor in plasma-cell biology, essential for long-lived plasma-cell survival and sustained antibody production^{188,189}. Its expression in IGG-high tumors suggests that robust humoral immunity may be a cornerstone of long-term immune surveillance. IL2RG, the common gamma chain shared by multiple interleukin receptors (IL-2, IL-7, IL-15, IL-21), is critical for lymphocyte development and homeostasis¹⁹⁰. Its inclusion in the IGG network highlights the importance of cytokine-mediated support of both B- and T-cell responses. POU2AF1 (OBF1) is a transcriptional co-activator regulating immunoglobulin gene expression and germinal center reactions, reinforcing the link between IGG and B-cell differentiation programs¹⁹¹.

CD27, a co-stimulatory receptor expressed on B and T cells, contributes to both memory B-cell formation and T-cell activation. Its presence within the CIGGs underscores the integrated nature of adaptive immunity, where B- and T-cell responses reinforce each other¹⁹². IGJ (JCHAIN) encodes the joining chain of polymeric immunoglobulins (IgM, IgA), enabling effective mucosal immunity and highlighting the systemic dimension of IGG-related biology¹⁹³. CD79A, a signaling component of the B-cell receptor complex, ensures proper B-cell activation and antigen-driven clonal expansion¹⁹⁴. Finally, PIM2, a serine/threonine kinase, has been implicated in regulating cell survival and lymphocyte metabolism, further supporting sustained

immune responses¹⁹⁵.

Notably, pairwise combinations of CIGGs provided stronger associations with survival than any single gene, suggesting that these genes do not act in isolation but rather in complementary fashion. For example, the combination of IL2RG (reflecting cytokine-driven lymphocyte persistence) and IGJ (immunoglobulin multimerization) may capture distinct yet synergistic dimensions of immune competence: cellular robustness and humoral breadth. Similarly, coupling BCMA (plasma-cell survival) with CD27 (immune memory and co-stimulation) integrates both effector persistence and long-term immunological readiness. These results suggest that immune resilience is best understood as a polygenic, multi-pathway phenomenon, rather than the effect of a single dominant gene.

From a biomarker perspective, these findings highlight opportunities to refine prognostic tools by focusing on CIGGs or their combinations. A smaller, gene-level panel derived from the IGG signature could provide a simplified yet powerful predictor of immune resilience and longevity. Such refinements could also enable mechanistic studies, as CIGGs may be easier to model *in vitro* or *in vivo* compared with the entire 14-gene IGG signature.

Translationally, several of these pathways are already of therapeutic interest. BCMA is a validated target in multiple myeloma, with CAR-T and bispecific antibodies demonstrating remarkable efficacy, raising the possibility that BCMA biology may also be exploitable in solid tumors. IL2RG-mediated cytokine signaling intersects with ongoing efforts to develop IL-7 and IL-15–based therapies to enhance T-cell persistence in cancer immunotherapy. CD27 agonists are under clinical evaluation to promote T-cell activity, and modulation of PIM2 kinase has been explored in hematological malignancies. These links underscore that the CIGGs are not only biomarkers of immune competence but also nodes of therapeutic opportunity that could, in principle, be manipulated to strengthen antitumor immunity or improve immune resilience in cancer survivors.

In summary, the identification of CIGGs provides mechanistic insight into the molecular underpinnings of the IGG signature. By converging on pathways that sustain humoral immunity, enable B–T cell cooperation, and preserve clonal diversity, CIGGs highlight how robust immune circuitry translates into improved patient survival. Their complementary action further suggests that future interventions may need to target immune networks rather than single genes to optimally enhance immune fitness and clinical outcomes.

Limitations

Our study has several limitations that should be considered when interpreting these findings. With respect to the immune-inflamed TME, we primarily relied on bulk transcriptomic and spatial proteomic data to infer immune composition and function. While these approaches revealed clear differences between IGG-high and IGG-low tumors, they cannot fully capture the dynamic interactions between immune and stromal cells. Multiplex imaging and functional assays will be needed to validate whether the reduced stromal remodeling and macrophage infiltration observed in IGG-high tumors directly contribute to improved therapeutic responses.

In relation to TLS biology, we demonstrated a strong association between IGG and TLS-like activity, even in the absence of histologically visible TLSs. However, TLS classification in our study was based on limited histopathological and spatial proteomic assessments. The degree of TLS maturity (early vs. germinal center-containing) could not be systematically evaluated, and this distinction may be clinically relevant, as mature TLSs are more strongly linked to favorable outcomes in other tumor types. Future studies with higher-resolution spatial approaches will be required to disentangle these nuances.

Regarding immune clonality, our BCR and TCR analyses showed greater polyclonality in IGG-high tumors, suggesting enhanced antigen recognition capacity. However, these analyses were performed in relatively small cohorts, and the single-cell RNA sequencing data were restricted to six tumors, which limits the generalizability of the repertoire findings. Although the results were supported by *in silico* validation in larger

external cohorts, the small sample size of the experimental datasets should be interpreted cautiously. Larger studies integrating bulk and single-cell repertoire analyses are needed to confirm these observations.

The link between IGG and longevity also warrants cautious interpretation. While the association was consistent across multiple breast cancer cohorts and extended to other tumor types, survival analyses were limited by the absence of detailed cause-of-death data. Although we focused on patients without recurrence, it remains possible that some deaths were due to undetected relapses or comorbid conditions unrelated to cancer. This uncertainty highlights the need for survivorship-focused datasets where cause of death and competing risks are systematically annotated.

Finally, the molecular underpinnings revealed by the CIGGs provide intriguing mechanistic hypotheses, but our evidence remains correlative. The suggestion that TNFRSF17, IL2RG, CD27, and other genes collectively sustain long-term immune resilience is compelling but requires functional validation. Experimental perturbation of these genes in preclinical models will be necessary to determine whether they actively drive immune competence or simply act as biomarkers of it.

Taken together, these limitations emphasize the importance of interpreting our findings as hypothesis-generating. Despite these caveats, the consistency of the results across independent datasets, multiple technologies, and analytic frameworks strengthens confidence that IGG captures fundamental features of the antitumor immune response and warrants further mechanistic and clinical investigation.

Clinical implications and future directions

Despite these limitations, our findings highlight the IGG signature as a multidimensional biomarker that integrates immune activation, TLS biology, repertoire diversity, and long-term survival. The fact that IGG is already incorporated into HER2DX means it can be deployed clinically today, providing oncologists with a scalable and standardized tool to refine prognosis and support treatment decisions in HER2-positive breast cancer. In particular, IGG may help identify patients who could benefit from

treatment de-escalation, sparing unnecessary toxicity, or conversely, those who may require novel therapeutic combinations to overcome immune-poor, stromal-dense microenvironments.

Prospective studies are now crucial. The ongoing DEFINITIVE trial (NCT06446882)¹⁹⁶, which randomizes patients with stage II–III HER2-positive disease across 7 countries and 44 hospitals to standard therapy versus HER2DX-guided therapy, represents a landmark effort to test the clinical impact of integrating IGG into treatment algorithms. The results of this study will help determine whether IGG, in conjunction with other HER2DX-derived scores, can safely guide systemic therapy choices in real-world practice.

A key next step will also be to deepen our understanding of TLS biology in breast cancer. While our study shows that IGG captures TLS-like immune activity, TLS themselves are heterogeneous, varying in number, cellular composition, and degree of maturity. Recent work by Dr. Elia Seguí and Dr. Esther Sanfeliu (ESMO Breast 2025; abstract 13P) has proposed a novel H&E-based TLS classification system, developed across more than 900 breast tumor samples. This represents one of the largest TLS evaluations conducted to date in breast cancer and distinguishes immature from mature TLS, correlating these categories with distinct gene expression programs, TIL levels, and IGG expression. Importantly, the study demonstrated that the HER2DX IGG signature displayed the strongest molecular correlation with TLS, achieving excellent predictive performance for TLS maturity (AUC = 0.83 for TLS 2b). At the same time, it confirmed that IGG, TLS, and TILs are interrelated yet non-redundant biomarkers, each contributing unique insights into tumor–immune biology. Building on these insights, future research should integrate molecular, histological, and spatial analyses of TLS to define their prognostic and predictive roles more precisely, as well as their potential as therapeutic targets. In particular, understanding TLS maturation could help identify patients most likely to benefit from immune checkpoint inhibitors and cell-based approaches such as tumor-infiltrating lymphocyte (TIL) therapy or CAR-T therapy, in which pre-existing immune niches may support sustained therapeutic responses.

In parallel, the extension of BCR and TCR repertoire analyses into blood represents a particularly promising future direction. Our tumor-based findings suggest that polyclonality is linked to enhanced immune fitness, and blood-based repertoire sequencing could provide a dynamic, minimally invasive readout of this biology. Profiling diversity at baseline could help identify patients with intrinsically stronger immune competence, while longitudinal monitoring during therapy and follow-up could track the expansion or contraction of tumor-reactive clonotypes. Such measurements may allow early detection of therapeutic efficacy, emerging resistance, or even relapse before clinical progression is apparent. Moreover, circulating BCR/TCR sequences could be mined for therapeutic purposes—for example, to identify tumor-specific antibodies or TCRs that can be developed into monoclonal antibodies, bispecifics, or engineered T-cell therapies.

Beyond HER2-positive breast cancer, the consistent association of IGG with survival across multiple cancer types suggests that it may function as a pan-cancer biomarker of immune fitness. This opens several opportunities:

- Patient stratification in immunotherapy trials: IGG could help identify patients most likely to respond to immune checkpoint inhibitors or other immunomodulatory strategies. This extends to cell-based therapies such as tumor-infiltrating lymphocyte (TIL) therapy and CAR-T cell therapy, where pre-existing immune competence may be critical for efficacy. IGG could serve as a tool to enrich for patients with inflamed, B/T cell-rich microenvironments more likely to sustain these therapies.
- Survivorship care: IGG may provide prognostic information not only about recurrence but also about long-term health outcomes, allowing survivorship programs to tailor monitoring and preventive interventions.
- Therapeutic innovation: Core IGG genes (CIGGs) may serve as targets for novel therapies designed to enhance B–T cell cooperation, plasma-cell survival, or immunoglobulin production, with the goal of boosting immune resilience in

cancer patients.

Looking ahead, the integration of IGG with other layers of immune data could enable the creation of composite “immune fitness scores.” By combining transcriptomic signatures (e.g., IGG), spatial metrics (e.g., TLS density and maturity), repertoire diversity (BCR/TCR clonality), and functional immune readouts (cytokine signaling, antigen presentation capacity), such scores could provide a holistic view of patient immune competence. These tools would allow oncologists to move beyond single-dimension biomarkers and toward a precision immuno-oncology framework that accounts for the complexity of tumor–immune interactions. Crucially, such an approach could guide not only checkpoint inhibitor therapy but also the next generation of cell-based therapies, including TIL and CAR-T approaches, ensuring that patients most likely to benefit are accurately identified.

Finally, future efforts must also consider equity and accessibility. While HER2DX provides a standardized platform for IGG measurement, access to genomic testing remains uneven across healthcare systems. Ensuring affordability and integration with digital pathology approaches such as automated TLS scoring will be essential to democratize immune biomarker testing globally. In parallel, IGG could be complemented by systemic, low-cost biomarkers of inflammation, such as circulating cytokines or hematological ratios (e.g., neutrophil-to-lymphocyte ratio), to broaden its utility in everyday practice and facilitate implementation in resource-limited settings.

Ultimately, the IGG signature exemplifies a new generation of biomarkers that do more than predict short-term treatment outcomes—they capture the deeper biology of immune resilience, linking cancer prognosis with broader principles of immunology and healthy aging. Its development and clinical translation may therefore impact not only breast cancer care but also the wider field of oncology, survivorship medicine, and public health. In this sense, the work presented in this thesis establishes IGG as both a biological marker of immune competence and a translational bridge toward precision immuno-oncology.

STUDY 2: ARI-HER2 PROJECT

In recent years, survival outcomes for patients with HER2-positive metastatic BC have significantly improved due to the introduction of novel HER2-targeted therapies³ such as T-DM1, T-DXd and tucatinib. Nevertheless, approximately 20% of patients continue to experience disease progression despite these advances, underscoring a persistent unmet clinical need^{41,42,52}. HER2-targeted CAR-T cell therapy has emerged as a promising alternative for these patients, offering a distinct mechanism of action that bypasses conventional resistance pathways.

While CAR-T therapies have demonstrated remarkable success in hematologic malignancies, their application in solid tumors have generally produced suboptimal results due to several challenges, including the difficulty to identify tumor-specific antigens, the immunosuppressive TME, poor T-cell infiltrations, antigen heterogeneity and physical barriers. However, studies in gastrointestinal¹⁶⁸, prostate¹³¹, and brain cancers^{133,134,173,174} have reported encouraging outcomes—including complete remissions and rapid tumor regressions—highlighting their strong potential in addressing challenging solid tumors.

Despite these promising advances, targeting HER2 with CAR-T cells remains particularly complex due to safety concerns observed in early clinical trials. In 2010, the NIH reported a fatal case involving a patient with metastatic colorectal cancer treated with a third-generation HER2-targeted CAR-T product. This construct included CD28 and 4-1BB co-stimulatory domains and a high-affinity scFv derived from trastuzumab (4D5)¹²⁸. The patient developed acute respiratory distress syndrome within minutes of infusion and subsequently died. Autopsy findings revealed extensive pulmonary inflammation and lymphocytic infiltration, although it remains unclear whether the cause was CRS or an on-target off-tumor effect. Importantly, the dose administered in the NIH study was exceptionally high (10^{10} cells).

More recent clinical studies using second-generation HER2-targeted CAR-T constructs have demonstrated improved safety profile¹⁴⁰. Notably, early-phase trial in patients

with HER2-positive sarcomas have shown objective responses, including a complete response in a pediatric patient¹³⁹. These findings support continued development of HER2-targeted CAR-T therapies, especially for patients with HER2-positive metastatic BC who remain in need of innovative treatment options.

ARI-HER2 construct

To address this need, we designed a CAR construct based on prior published experience. Unlike the murine scFv used in sarcoma study, our construct incorporates a humanized 4D5.5 scFv derived from trastuzumab. This variant differs from the NIH 4D5 by two point mutations that reduce binding affinity: one in the light chain (Tyr55 → Glu in CDR2) and one in the heavy chain (Tyr102 → Val in CDR3). These modifications aim to reduce off-tumor toxicity while maintaining sufficient anti-tumor activity. Whereas the NIH CAR was a third-generation construct featuring dual co-stimulatory domains, ARI-HER2 is a second-generation CAR incorporating a CD8α hinge and transmembrane domain, along with 4-1BB and CD3ζ intracellular signaling domains. The use of 4-1BB is associated with enhanced CAR-T cell persistence and reduced exhaustion compared to CD28-based constructs¹⁹⁷.

Other anti-HER2 CARs tested in sarcoma and glioblastoma have also been second-generation, but typically utilized CD28 as the co-stimulatory domain, which is associated with stronger initial activation but reduced long-term persistence¹⁹⁸. The choice of co-stimulatory domain is critical, as it influences not only the kinetics of T-cell activation but also the durability of the anti-tumor response and the risk of adverse events.

Optimal treatment strategies for solid tumors—including preconditioning regimens, administration routes, and dosing schedules—remain undefined, complicating direct comparisons across clinical trials. Preconditioning chemotherapy, typically with lymphodepleting agents such as cyclophosphamide and fludarabine, is known to enhance CAR-T cell expansion and persistence, but its role in solid tumors is still being refined. Moreover, regional delivery methods (e.g., intratumoral or intraventricular

infusion) are being explored to improve CAR-T cell trafficking and reduce systemic toxicity.

Nevertheless, aside from the NIH study, which employed exceptionally high cell doses (approximately 30 times higher than the dose we would administer to a patient weighing 70 kg), the dosing regimens used in other HER2-targeted CAR-T trials have been comparable to ours, supporting the feasibility and safety of our approach.

Strategies to minimize tonic signaling

A well-recognized limitation of certain CAR constructs is the presence of tonic signaling, a form of ligand-independent activation that can be detected during primary T cell expansion¹⁹⁹. In CARs incorporating the 4-1BB costimulatory domain, this phenomenon has been associated with a 70% reduction in proliferation during ex vivo expansion, increased apoptosis, and progressive downregulation of CAR expression¹⁴⁶. Consequently, selecting CAR designs that minimize tonic signaling is essential to achieve robust, antigen-specific responses while preserving safety and functionality.

To address these challenges, we explored multiple strategies aimed at reducing tonic signaling, with the goal of optimizing T cell manufacturing and enhancing functional potency. We focused on the pharmacological inhibition of key signaling pathways implicated in CAR activation, including LCK, AKT, PI3K, LFA-1, and FASL. Dasatinib, an LCK inhibitor, interferes with early CAR signaling by blocking phosphorylation of CD3 ζ and ZAP70, thereby temporarily silencing CAR activation without compromising cell viability or expansion^{161,163,200}. Similarly, inhibition of PI3K using idelalisib or AKT also conferred benefits, such as increased transduction efficiency, a higher proportion of less differentiated CAR-T cells, and reduced expression of exhaustion markers such as PD-1 and Tim-3^{149,201–203}. Inhibition of LFA-1 prevented its interaction with ligands including ICAM-1, ICAM-2, and ICAM-3, disrupting leukocyte adhesion and T cell activation²⁰⁴. FasL, which is upregulated in activated CAR-T cells, acts as a negative regulator by inducing apoptosis through the Fas–FasL pathway when signaling becomes excessive, contributing to CAR-T cell dysfunction and elimination through

activation-induced cell death (AICD), ultimately compromising persistence and antitumor efficacy^{205–207}.

Among all the inhibitors tested, dasatinib at 50 nM emerged as the most effective, significantly improving T cell yield, reducing apoptosis, and preventing CAR-T cell loss during the expansion phase. Continuous dasatinib exposure from day 3 to day 9 did not provide additional benefit compared to short-term exposure from day 3 to day 4 in terms of cell expansion, CAR expression, or functional activity. Transcriptomic analysis using the nCounter platform revealed no significant differences between these two treatment durations. Therefore, to preserve the quality of the final cell product and minimize unnecessary manipulation, the short-term exposure protocol was selected.

To better understand the molecular impact of dasatinib during ex vivo expansion, we performed transcriptomic profiling comparing HER2-BBz CAR-T cells cultured with or without dasatinib to UTD T cells. CAR-T cells cultured without dasatinib exhibited a markedly higher number of differentially expressed genes, indicative of strong transcriptional activation likely driven by CAR-associated tonic signaling. In contrast, the presence of dasatinib significantly reduced the transcriptomic alterations, suggesting effective suppression of this basal activation. Apoptosis-related genes were notably downregulated in dasatinib-treated cells, while upregulated in untreated CAR-T cells. This included key pro-apoptotic genes such as *GZMB*, *GZMA*, *LTA*, *JUN*, and *CXCL10*. *GZMB* (Granzyme B) mediates perforin-dependent cytotoxicity through activation of caspases 3 and 7 and can also trigger mitochondrial damage via Bid activation and subsequent cytochrome c release²⁰⁸. Acting in a complementary manner, *GZMA* (Granzyme A) induces cytotoxicity through a non-classical, caspase-independent pathway²⁰⁹. *LTA* (Lymphotoxin Alpha) functions as a proapoptotic cytokine that binds TNFR family receptors, recruits TRADD/FADD, and activates caspase-8, although in certain contexts it can instead activate NF-κB and promote survival²¹⁰. *JUN*, a dual-function transcription factor, can adopt a proapoptotic role by inducing the expression of FasL and Bim as part of the AP-1 complex²¹¹. Finally, *CXCL10* does not directly cause cell death but promotes tissue infiltration by cytotoxic

T cells and NK cells, thereby facilitating apoptosis indirectly²¹². Pathway enrichment analysis supported these findings, confirming activation of apoptotic pathways in the absence of dasatinib and their attenuation upon treatment, reinforcing the role of dasatinib in protecting against activation-induced apoptosis during CAR-T cell manufacturing.

A limitation of this study is the restricted gene set targeted by the Nanostring panel, which may constrain the detection of broader transcriptomic differences. To address this, future studies could employ more comprehensive approaches such as bulk or single-cell RNA-seq, complemented by qRT-PCR validation and functional assays. These strategies would enable the identification of wider transcriptomic changes, reveal the cellular subpopulations involved, and link molecular alterations to phenotypic outcomes.

Importantly, when CAR-T cell expansion was performed under GMP-compliant conditions using the CliniMACS Prodigy automated cell processing system did not elicit detectable tonic signaling, and therefore dasatinib will not be used in the clinical setting. Nevertheless, incorporating dasatinib in our studies enabled robust preclinical evaluation and sufficient cell yields for in vivo studies. Moreover, this protocol is currently being used in our laboratory to expand other CAR constructs that present tonic signaling or can induce T-cell fratricide, such as CARs targeting CD7 or CD70.

Beyond pharmacological interventions, several non-pharmacological strategies have also been explored to mitigate tonic signaling, although these were not assessed in the present study. These include hinge region modifications, scFv engineering to enhance molecular stability, incorporation of an internal ribosome entry site (a genetic element that allows controlled downstream gene expression) upstream of the LTR promoter, and transduction with self-inactivating lentiviral vectors to limit excessive CAR expression²¹³. In a previous project conducted in our lab, various intracellular and transmembrane domains were tested. Although some combinations showed reduced tonic signaling, the ARI-HER2 configuration remained the most effective overall.

To assess the potential clinical applicability of this approach, we generated non-clinical batches of HER2-BBz CAR-T cells using PBMCs isolated from two patients with HER2-positive BC. The results confirmed that patient-derived T cells could be efficiently transduced with the HER2-BBz construct, and that dasatinib exposure during ex vivo expansion did not impair CAR-T cell functionality.

ARI-HER2 safety profile

Once the CAR-T manufacturing process had been optimized and its functionality validated, we proceeded to assess the safety profile of the final product. *In vitro* assays were performed using primary cells with low HER2 expression and the MDA-MB-468 cell line genetically modified to express different HER2 levels. These experiments included a direct comparison between our HER2-BBz CAR and the high-affinity HER2-28BBz CAR developed by the NIH¹²⁸. The results demonstrated that HER2-BBz CAR selectively eliminated tumor cells overexpressing HER2, while sparing healthy cells expressing physiological levels of the target antigen. However, when performing a side-by-side comparison between ARI-HER2 and UTD T cells, a small degree of CAR activation was observed. This finding prompted a more conservative clinical approach, including dose fractionation and stricter clinical monitoring protocols.

To further investigate this selectivity *in vivo*, and as part of another project in our laboratory, the modified MDA-MB-468 cell line with varying HER2 densities was employed. In this context, HER2-BBz CAR was again compared to the NIH high-affinity construct (B.Marzal EHA 2022 abstract). The results suggested that HER2-BBz CAR can effectively discriminate between high and physiological HER2 expression, achieving comparable antitumor efficacy with reduced off-tumor toxicity, thus supporting a broader therapeutic window. Nevertheless, this high level of specificity may also represent a potential limitation. In tumors with heterogeneity, HER2-BBz CAR-T cells might fail to eliminate subclones with low or absent HER2 expression. To mitigate this risk in the clinical trial, an initial tumor biopsy will be performed, and only patients with HER2 overexpression confirmed by 3+ immunohistochemistry will be eligible for inclusion. Moreover, we hypothesize that some degree of antitumor activity may still

occur in the context of tumor heterogeneity. Cytokine release following CAR activation or the liberation of other antigens could potentially recruit and activate additional immune cells within the tumor microenvironment, a phenomenon known as epitope spreading, which may contribute to the elimination of HER2-low or HER2-negative subclones²¹⁴.

Toxicological concerns were raised by the Spanish Agency of Medicines and Medical Devices (AEMPS) regarding potential HER2 overexpression in non-tumoral contexts that could pose safety risks for HER2-BBz CAR-T cells, especially considering their expected long-term persistence in patients. Two scenarios were considered: (1) inflammatory conditions such as colitis or hepatitis, and (2) potential modulation of HER2 expression in healthy tissues following chemotherapy.

In the first scenario, analysis of inflammatory tissue samples from colitis and hepatitis revealed only minimal HER2 protein expression in a small fraction of cases. Transcriptomic profiling using a NanoString panel confirmed that *ERBB2* mRNA levels in these tissues were comparable to those observed in HER2-negative BC and markedly lower than in HER2-positive tumors, suggesting that HER2 expression under the inflammatory conditions evaluated remains well below tumor-associated levels. Although the number of cases analyzed was limited, these findings provide preliminary reassurance, and future studies should consider expanding the sample size to confirm these observations.

Regarding the second concern, a literature review found no evidence of chemotherapy-induced HER2 overexpression in healthy tissues; instead, multiple studies reported reductions in tumor HER2 expression following cytotoxic treatment, particularly in combination with anti-HER2 therapies. One study involving 21,755 BC patients found that the 21.4% of those with HER2-positive tumors experienced a reduction in HER2 expression after receiving chemotherapy and neoadjuvant anti-HER2 therapy²¹⁹. Other studies have reported similar results both with BC patients and in HER2-positive gastric cancer patients treated with pre-operative chemotherapy²²⁰. Consistent with these findings, our analysis of plasma HER2 levels in HER2-positive BC patients showed

significantly elevated baseline concentrations that decreased after chemotherapy and remained low during disease control. In one patient, HER2 levels rose upon tumor progression and declined again after initiation of trastuzumab-deruxtecan, mirroring changes in tumor burden. Although plasma HER2 levels are not currently used as a clinical biomarker for disease monitoring, these observations suggest that further studies could explore their potential utility as indicators of treatment response or resistance.

The potential modulation of HER2 expression in healthy tissues as a consequence of chemotherapy treatment has not been formally studied. Addressing this specific question would require paired biopsies of healthy tissues before and after chemotherapy, a procedure that is not considered ethically acceptable due to the additional risk it poses to patient safety. Collectively, these results indicate that the inflammatory conditions examined, as well as chemotherapy, do not induce clinically relevant HER2 upregulation in non-tumoral tissues. The consistently low expression levels observed across both scenarios provide robust experimental evidence that addresses the safety concerns raised by AEMPS. These results support the continued development of HER2-BBz CAR-T cells from a toxicological standpoint, reinforcing the rationale for their clinical application and the design of a safety-focused first-in-human trial.

Clinical trial

Taking into account the data presented above, we propose initiating a multicenter, first-in-human clinical trial for patients with HER2-positive metastatic BC who have progressed after at least two prior lines of treatment, including T-DXd. This study represents a pioneering effort in Europe for HER2-targeted CAR-T cell therapy in BC and is supported by a €1 million grant awarded by the *Asociación Española contra el Cancer* (AECC), ECAEC235235DELG.

Based on published clinical trials that report dosing strategies, efficacy outcomes, and adverse events, the selected dose escalation for our study ranges from 1.5 to 4.5×10^6 CAR-T cells/kg administered intravenously. The trial adopts a conservative dosing

strategy with fractionated administration (10% of the total dose on day 0, 30% on day +3, and 60% on day +7) designed to minimizing the risk of on-target off-tumor toxicity and cytokine release syndrome (CRS).

Given the potential risks associated with HER2 targeting, systematic procedures will be implemented to ensure early identification and management of toxicities. These procedures are designed to complement scheduled study assessments, promote consistency across participating sites, standardize reporting, and enable timely clinical intervention. Cardiotoxicity will be monitored through serial measurements of high-sensitivity troponin and NT-proBNP, echocardiograms and electrocardiograms. Pulmonary toxicity will be monitored through pulse oximetry, spirometry and high-resolution chest CT scans. Digestive toxicity will be assessed through regular clinical evaluations and laboratory testing to detect and manage treatment-related diarrhea, gastrointestinal adverse events, and hepatic or pancreatic toxicity. Renal function will be monitored at each scheduled blood test, and bone mineral density will be evaluated through serial densitometry to identify potential bone loss. Systematic clinical anamnesis and physical examination at each visit will facilitate the detection of other adverse events potentially related to off-tumor HER2 targeting. To ensure uniformity across institutions, all site personnel will receive training focused on the early detection of toxicities. The electronic case report form (eCRF) will include dedicated fields for cardiac biomarkers, echocardiographic data, pulse oximetry, and pulmonary assessments to support standardized documentation and analysis.

Expanding the application of HER2DX to the metastatic setting may provide clinically valuable insights with significant therapeutic implications. Among the components of HER2DX, the *ERBB2* mRNA score has already demonstrated prognostic value in patients with advanced HER2-positive BC treated with trastuzumab, pertuzumab, and taxane-based regimens, where higher *ERBB2* expression was associated with prolonged PFS and OS⁷⁰. This suggests that the *ERBB2* score could be leveraged as a biomarker to refine patient selection for emerging HER2-directed therapies, including CAR-T cells, by identifying tumors that remain strongly dependent on HER2 signaling.

In the ARI-HER2 clinical trial—the first study in Europe to evaluate a HER2-targeted CAR-T cell therapy in BC—only patients with HER2 IHC 3+ tumors will be eligible, ensuring the inclusion of individuals with unequivocal HER2 overexpression. HER2DX testing will provide an additional molecular layer of characterization. In parallel, the IGG immune signature, previously identified as a determinant of therapeutic response and long-term survival in early-stage HER2-positive disease, will also be assessed in trial samples. This will allow the exploration of how the immune microenvironment, particularly B-cell-driven IGG biology, influences the efficacy of novel immune-based therapies such as CAR-T cells. Collectively, integrating HER2DX analyses into ARI-HER2 not only strengthens the biomarker component of the study but also establishes a direct translational bridge between immune profiling and therapeutic innovation in HER2-positive BC.

In summary, ARI-HER2— designed to exhibit lower toxicity compared to high affinity anti-HER2 CAR-T cells— will be administered at an initial dose of 1.5×10^6 CAR-T cells/kg in cohort 1, with the first fraction corresponding to 10% of this dose. It is important to note that the fatal toxicity previously reported with the high-affinity CART-HER2 construct occurred at a substantially higher dose of 10^{10} cells, which is approximately 30 times greater than the dose that would be administered to a 70 kg patient in our trial. The administration of the second and/or third fractions will be withheld in the presence of any of the following: grade 3 or higher CRS, grade 2 or higher ICANS, clinical or microbiological evidence of active infection, or signs of potentially severe on-target off-tumor toxicity.

Taken together, the combination of rigorous preclinical validation, strategic patient selection, and a conservative clinical design aims to maximize therapeutic benefit while ensuring patient safety. This approach marks a significant step forward in the development of CAR-T cell therapies for solid tumors, offering a framework for safe and effective translation of this technology into clinical practice.

CONCLUSIONS

1. IGG-high HER2-positive tumors display an immune-inflamed microenvironment enriched with plasma cells, T cells, and antigen-presenting cells, together with increased B- and T-cell polyclonality and reduced immune suppression and fibrosis.
2. The IGG signature provides a scalable and minimally invasive approach to infer the presence of tertiary lymphoid structures from bulk transcriptomic data, improving diagnostic accuracy.
3. The IGG signature emerges as a reliable biomarker for longevity in breast cancer survivors, with potential applicability to other cancer types. These findings support the development of more personalized and effective immune-based therapeutic strategies that address both recurrence risk and long-term outcomes.
4. Combining two complementary immune genes further enhanced predictions of longevity and revealed robust T-cell-mediated antitumor immune responses, highlighting the therapeutic relevance of capturing immune activation in HER2-positive disease.
5. Short-term exposure to dasatinib early after T-cell activation effectively reduces tonic signaling in 4-1BB-based CAR-T cells, enhancing T-cell expansion increasing CAR expression.
6. Patient-derived T cells were efficiently transduced with the HER2-BBz construct, and their expansion in the presence of dasatinib maintained full functional activity.

7. HER2-BBz CAR-T cells selectively discriminated between high and physiological HER2 expression, achieving potent antitumor efficacy with reduced off-tumor toxicity, thereby broadening the therapeutic window.
8. These findings supported the initiation of the ARI-HER2 clinical trial, representing a significant advancement in the clinical development of CAR-T therapies for solid tumors.
9. Together, these two projects demonstrate how dissecting the immune microenvironment can inform the design of innovative immunotherapies, advancing both prognostic tools and therapeutic strategies in HER2-positive breast cancer.

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During the preparation of this work the author used ChatGPT 4 to improve language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

APPENDIX

IGG ARTICLE

ORIGINAL ARTICLE

Linking tumor immune infiltration to enhanced longevity in recurrence-free breast cancer

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Background: The infiltration of tumor-infiltrating B cells and plasma cells in early-stage breast cancer has been associated with a reduced risk of distant metastasis. However, the influence of B-cell tumor infiltration on overall patient survival remains unclear.

Materials and methods: This study explored the relationship between an antitumor immune response, measured by a 14-gene B-cell/immunoglobulin (IGG) signature, and mortality risk in 9638 breast cancer patients across three datasets. Associations with tumor subtype, stage, and age were examined. IGG was characterized using spatial GeoMx profiling and single-cell RNA sequencing, and its relationship with tertiary lymphoid structures (TLSs) was evaluated. The predictive value of each of the 14 IGG genes for B-cell receptor (BCR) and T-cell receptor (TCR) clonality and longevity was also assessed, along with its association with longevity in other cancer types.

Results: High IGG signature expression was significantly associated with a 41%–47% reduction in death risk in breast cancer survivors ($P < 0.001$), regardless of age, tumor stage, or subtype. Similar associations were observed in other cancers, including melanoma. In breast cancer, the IGG signature was significantly linked to overall survival without relapse in patients aged 41–70 years at diagnosis. Additionally, IGG expression correlated with the presence of TLSs and higher B- and T-cell polyclonality. A specific subset of seven IGG genes strongly correlated with BCR and TCR clonality, with predictive power for identifying clonality and improved longevity, especially when combining two of these genes.

Conclusions: This study uncovers a significant link between immune gene expression in tumors and extended longevity in breast cancer survivors, even in the absence of recurrence. The IGG signature, particularly its key gene subset, emerges as a powerful marker of sustained antitumor immunity and overall patient fitness. These findings pave the way for personalized treatment strategies that enhance both survival and long-term health outcomes.

Key words: breast cancer, immune gene expression, longevity, clonality

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INTRODUCTION

The 14-gene B-cell/immunoglobulin (IGG) signature has emerged as a robust prognostic marker in early-stage breast cancer.¹ It comprises genes involved in lymphocyte progenitor maturation, activation, differentiation, immunoglobulin production, chemotaxis, and lymphocyte activity regulation; it was first identified through unsupervised clustering of 550 node-negative breast tumors,¹ and has been externally validated across diverse clinical cohorts involving over 1000 patients.^{2,3} This signature is incorporated into the 27-gene HER2DX genomic assay, designed for early-stage human epidermal growth factor receptor 2 (HER2)-positive disease.^{1,2,4-9} The HER2DX assay integrates clinicopathological and genomic data to generate two distinct scores, which predict disease-free survival and the likelihood of achieving a pathological complete response (pCR) following neoadjuvant trastuzumab-based therapy,² and has been shown to have statistically significant association with both event-free survival and overall survival (OS).⁵ Notably, within the HER2DX framework, a high IGG signature expression correlates with increased pCR rates and improved survival outcomes.²⁻⁵ High IGG signature expression is also predictive of pCR, event-free survival, and OS in patients with early-stage triple-negative breast cancer (TNBC).^{9,10}

Adaptive immunity, known for its high specificity and ability to provide long-term antigen-specific memory, might help explain the strong correlation between the IGG signature, breast cancer recurrence, and OS.^{2,5} However, the predictive power of immune tumor infiltration for longevity, especially in the absence of disease recurrence, remains uncertain. Bridging this knowledge gap could substantially deepen our understanding of the immune system's persistent impact on patient outcomes, potentially leading to improved prognostic tools and therapeutic strategies.

MATERIALS AND METHODS

Patient datasets

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC)¹¹ ($n = 1904$) and The Cancer Genome Atlas (TCGA)¹² ($n = 1082$) breast cancer datasets and pan-cancer TCGA datasets [i.e. cervix ($n = 294$), head and neck ($n = 515$), lung ($n = 510$), melanoma ($n = 443$), and sarcoma ($n = 253$)] were sourced from the cBioPortal for Cancer Genomics (<http://cbioportal.org>),¹¹ while data from the most recent version of The Sweden Cancerome Analysis Network (SCAN-B)^{13,14} ($n = 6652$) dataset were obtained from Mendeley Data (<https://data.mendeley.com/datasets/yxxtxn4nmd>). Except for the competing risk analyses, patients who experienced breast cancer recurrence were systematically excluded from both datasets. The final analysis comprised 1133 patients from METABRIC, 4470 patients from SCAN-B, and 936 patients with breast cancer from TCGA, ensuring a cohort that did not encounter recurrence during the follow-up period. The median follow-up in these patients

was 96.9 months in SCAN-B, 185.0 months in METABRIC, and 130.0 months in TCGA. Gene expression was obtained from all datasets. Additionally, *TP53*, *PIK3CA*, and *RB1* somatic mutation data (i.e. missense, inframe, splicing, truncating) were downloaded for TCGA and METABRIC. Normal breast tissue gene expression data from 168 women were downloaded from GTEx (<https://gtexportal.org/home/>). RNA sequencing (RNA-seq) and B-cell receptor (BCR) and T-cell receptor (TCR) repertoire metrics from the CALGB-40601 neoadjuvant HER2-positive study were obtained from a previously published study.¹⁵

In silico gene expression analysis

Z-scores (METABRIC, TCGA) or log2-transformed (SCAN-B) normalized gene expression values were employed for single genes and for deriving the 14-gene IGG signature, depending on the available gene expression data for each dataset (refer to Supplementary Table S8, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). The Prediction Analysis of Microarray 50 (PAM50) risk of recurrence (ROR) was also determined for METABRIC and TCGA.

Core IGG genes

Genes consistently associated with longevity across METABRIC, SCAN-B, and TCGA datasets were identified as core IGG genes (CIGGs). We further investigated whether the combined assessment of pairs of CIGGs enhanced longevity prediction compared with individual genes. Various mathematical operations, including addition, subtraction, multiplication, and ratio, were employed to combine the expressions of two CIGGs.

Clinical samples

To study the immune component of breast cancers with different levels of IGG signature, 132 formalin-fixed paraffin-embedded (FFPE) samples from patients with early-stage breast cancer treated at Hospital Clinic of Barcelona ($n = 122$) and Institut Català d'Oncologia ($n = 10$) were selected, including 35 hormone receptor-positive (HR+)/HER2-negative (HER2-), 84 HER2-positive, and 13 TNBC samples. The proportion of tumor-infiltrating lymphocytes (%TILs) was determined on hematoxylin-eosin (H&E) slides according to International TILs Working Group Guidelines.¹⁶ The presence of tertiary lymphoid structures (TLSs) was defined as spatially organized, non-encapsulated areas of immune cell aggregates on H&E slides. Additionally, six biopsies from patients with untreated HER2-positive breast cancer were obtained to conduct single-cell RNA-seq.

Gene expression analysis of clinical samples

RNA was extracted from FFPE tumor diagnostic samples using the High Pure FFPE RNA Isolation Kit (Roche, Indianapolis, IN). One to five 10- μ m FFPE slides depending on tumor cellularity were used for each tumor sample, and macrodissection was carried out, when needed, to avoid

normal tissue contamination. A minimum of 100 ng of total RNA was analyzed on the nCounter platform (NanoString Technologies, Seattle, WA) using a 192-gene custom panel, including 14 genes of the IGG signature, which was determined using R software v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria).

GeoMx DSP data acquisition and analysis

FFPE tumor biopsies from 135 patients were stained with fluorescently labeled CD45 and PanCK antibodies, DAPI (4',6-diamidino-2-phenylindole), and a multiplexed panel of protein antibodies that contained a photocleavable indexing oligonucleotide, enabling subsequent readouts. Regions of interest were selected on the GeoMx DSP platform (NanoString Technologies¹⁷), segmented according to CD45 and PanCK staining and illuminated using UV light. Released indexing oligonucleotides from each area of interest (AOI) were collected and deposited into designated wells on a microtiter plate, allowing for well indexing of each AOI during nCounter (NanoString Technologies) readout. For each tissue sample, counts for each marker were obtained from an average of 4.44 (range 1–12) CD45-positive (CD45+) AOIs. Raw protein counts for each marker in each AOI were generated using nCounter. The raw counts were normalized with the AOI surface area. All normalized counts were log2 transformed.

TCR and BCR sequencing

DNA from FFPE tumor samples with different IGG signature levels was purified with the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions and BCR and TCR were sequenced from 23 and 33 DNA samples, respectively, using the immunoSEQ Assay (Adaptive Biotechnologies, Seattle, WA). The somatically rearranged *Homo sapiens* TCR locus complementarity-determining region 3 (CDR3) and BCR immunoglobulin heavy chain (IgH) locus CDR3 were amplified from DNA samples using a two-step, amplification bias-controlled multiplex PCR approach. CDR3 and reference gene libraries were sequenced on an Illumina (San Diego, CA) instrument according to the manufacturer's instructions. Raw sequence reads were demultiplexed according to Adaptive's proprietary barcode sequences. Demultiplexed reads were further processed to remove adapter and primer sequences, and identify and remove primer dimer, germline, and other contaminant sequences. The filtered data were clustered using both the relative frequency ratio between similar clones and a modified nearest-neighbor algorithm, to merge closely related sequences to correct for technical errors introduced through PCR and sequencing. The resulting sequences allowed annotation of the V, D, and J genes and the N1 and N2 regions constituting each unique CDR3 and the translation of the encoded CDR3 amino acid sequence. Gene definitions were based on annotation in accordance with the IMGT database (www.imgt.org). The set of observed biological TCR and BCR IgH CDR3 sequences was normalized to correct for residual multiplex PCR

amplification bias and quantified against a set of synthetic CDR3 sequence analogues. Data were analyzed using the immunoSEQ Analyzer toolset.

Single-cell RNA sequencing

Fresh core biopsies obtained from six patients with HER2-positive breast cancer were collected by ultrasound-guided breast biopsy and the tissue (~3–5 mm³) was supplied in 5 ml of RPMI medium. Tumor fragments were dissociated using enzymatic digestion (Human Tumor Dissociation Kit, Miltenyi, Bergisch Gladbach, Germany). Then, CD45+ cells were isolated using Human CD45 TIL MicroBeads (Miltenyi) and stored in phosphate-buffered saline containing 0.005% bovine serum albumin. CD45+ cells were processed with the 10x Genomics Chromium Controller (10x Genomics Inc.) and single-cell gene expression and TCR/BCR libraries were produced with the Chromium Single Cell 5' Library assay (10x Genomics Inc., Newark, CA), sequenced on an Illumina NovaSeq6000 (100 cycle kit). Sequencing reads were aligned with Cell Ranger Single Cell Software (version 6.1.1, 10x Genomics Inc.) and mapped against the human GRCh38 reference genome (GENCODE v32/Ensembl 98). Quality control, cell annotation, and cluster identification were carried out based on rationale.¹⁸

Statistical analysis

Cumulative incidence functions were used to estimate death without recurrence in a competing risk setting using recurrence as a competing event. Fine and Gray competing risk regression was used to obtain a sub-distribution hazard ratio with 95% confidence interval (CI). In the sensitivity analysis of the subset of patients without recurrence, Kaplan–Meier curves were used to estimate survival outcomes and the log-rank test was used for statistical comparisons. Univariate and multivariable Cox regression and logistic regression models were employed, with the goodness of fit of each model assessed through the chi-square statistic obtained from a likelihood ratio test. The differences in variable distribution were explored by chi-square test. Correlations between two variables were evaluated using the Pearson method. Unpaired t-tests and analyses of variance were employed to ascertain the comparative analysis of numerical variables among different groups. A two-sided α of 0.05 was set as the significance level for all statistical analyses. No data imputation was carried out. All statistical analysis were carried out using the R software.

RESULTS

IGG, age, and longevity

We investigated the association between the IGG signature and OS in patients with early-stage breast cancer who have not experienced a relapse, a condition we defined as 'longevity' (Figure 1A). Leveraging comprehensive gene expression and clinical data from three distinct cohorts of

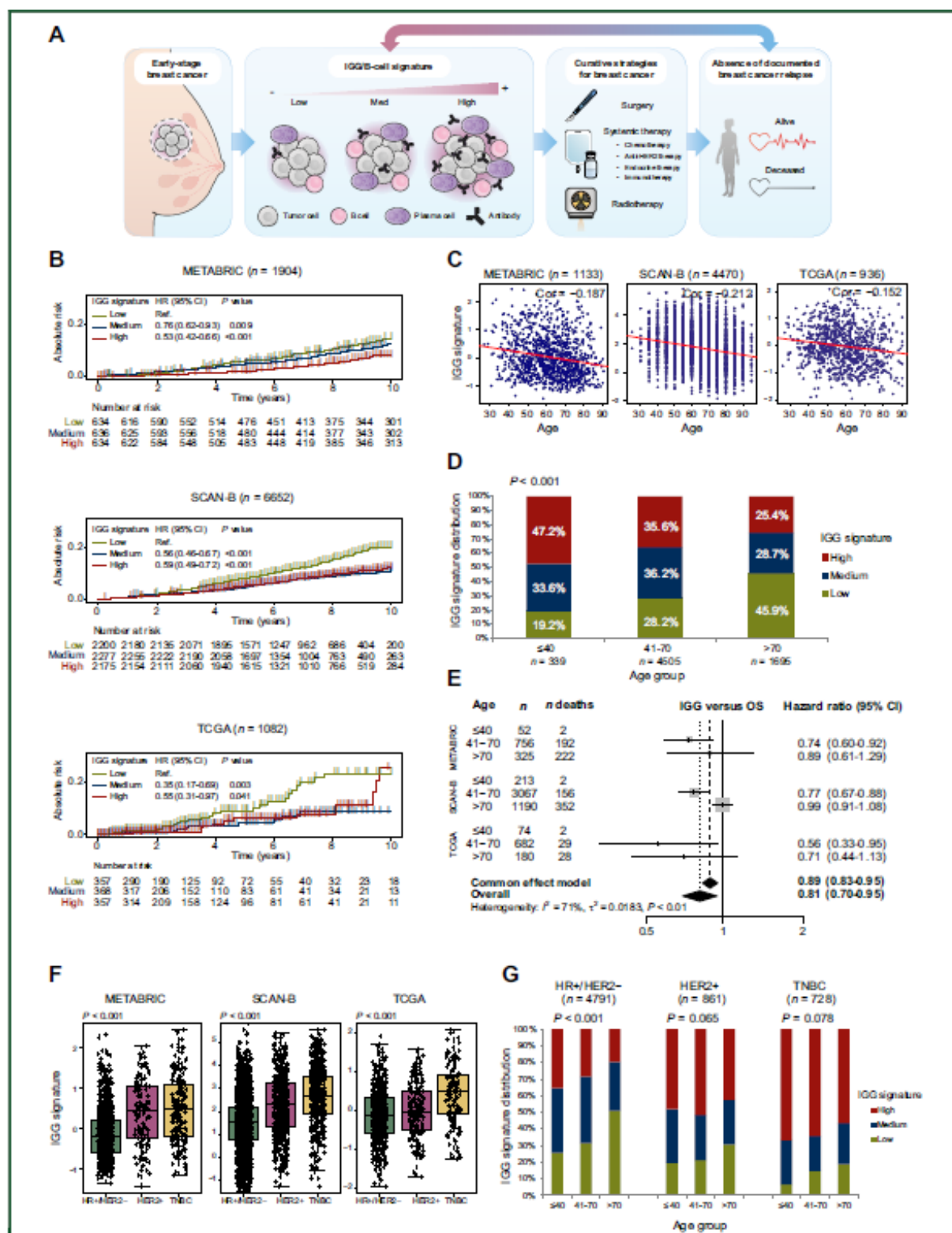


Figure 1. Assessment of the 14-gene IGG signature for predicting longevity in breast cancer survivors without a documented recurrence. (A) Schematic representation of the study approach. (B) Cumulative risk plots with censored recurrences illustrating the association of IGG expression with OS in all patients from METABRIC (n = 1904), SCAN-B (n = 6652), and TCGA datasets (n = 1082). (C) Correlation analysis depicting the relationship between the IGG signature and age in all patients without documented breast cancer recurrence during follow-up in METABRIC (n = 1133), SCAN-B (n = 4470), and TCGA datasets (n = 936).

9638 patients with early-stage breast cancer and long-term follow-up (i.e. METABRIC,¹¹ SCAN-B,^{13,14} and TCGA,¹² Supplementary Table S1, available at <https://doi.org/10.1016/j.esmoop.2024.104109>), we observed a significant association between IGG expression and OS without recurrence both treating recurrences as a competing event (Figure 1B) and excluding recurrences (Supplementary Figure S1, available at <https://doi.org/10.1016/j.esmoop.2024.104109>), independently from breast cancer subtype, tumor stage, or nodal status (Supplementary Table S2, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). This association persisted in patients with a very low risk of relapse (Supplementary Figure S1, available at <https://doi.org/10.1016/j.esmoop.2024.104109>), and patients with >5 years of follow-up without a documented recurrence. Moreover, we analyzed the relationship between the PAM50 ROR and the IGG signature in the TCGA and METABRIC datasets. Differences in PAM50 ROR distribution were observed across IGG groups, showing that IGG-high tumors are enriched in both ROR-high and ROR-low categories, with a lower proportion of ROR-medium, particularly within HR+/HER2- breast cancer (Supplementary Figure S2A, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). From a longevity perspective, the PAM50 ROR was not significantly associated with OS in recurrence-free breast cancer survivors (Supplementary Figure S2B, available at <https://doi.org/10.1016/j.esmoop.2024.104109>), whereas the IGG signature remained independently associated with survival in this population, even after adjusting for ROR (Supplementary Table S3, available at <https://doi.org/10.1016/j.esmoop.2024.104109>).

A weak negative correlation between the IGG signature and age was observed (Figure 1C and D). However, the IGG signature was significantly associated with OS without a relapse in patients aged 41-70 years at diagnosis, which represented 66.7%-72.9% of all patients (Figure 1E). Across breast cancer subtypes, the expression of the IGG signature was higher in HER2-positive and TNBC than in HR+/HER2- (Figure 1F); however, 26.2% of HR+/HER2- tumors were IGG-high. Finally, IGG decreased with age across breast cancer subtypes, especially in patients >70 years of age with HR+/HER2- disease (Figure 1G). Additionally, to address this, we analyzed the association of *TP53*, *PIK3CA*, and *RB1* mutations with IGG groups across breast cancer subtypes in the TCGA and METABRIC datasets (Supplementary Figures S3 and S4, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). Interestingly, *TP53* mutations were more frequently observed in IGG-high tumors. Despite this association, the IGG signature remained independently and significantly associated with OS in recurrence-free patients in a multivariable analysis

(Supplementary Table S4, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). In contrast, no significant differences were found in the frequency of *PIK3CA* or *RB1* mutations across IGG groups (Supplementary Figures S3 and S4, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). Of note, the IGG signature assessed in normal breast tissue was not associated with age (Supplementary Figure S5, available at <https://doi.org/10.1016/j.esmoop.2024.104109>).

IGG, spatial profiling, and TLSs

The IGG signature is measured from bulk RNA obtained from a tumor sample.² To gain deeper insights of the tumor microenvironment (TME) and its relationship with the IGG signature, we carried out GeoMx spatial profiling of stroma from 132 patients with breast cancer, which had been previously categorized as IGG-high, IGG-medium, and IGG-low, according to pre-established cut-offs of the HER2DX genomic test (Figure 2A and Supplementary Figure S6, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). We selected 458 CD45+ TME AOs, and we measured 42 immune-related proteins (Supplementary Data S1, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). Compared with IGG-medium and IGG-low tumors, IGG-high tumors showed increased expression of plasma cells (CD27), activated T cells, or antigen-presenting cells (programmed death-ligand 1 and CD40) and decreased expression of M2-like macrophages (CD163) and stromal fibronectin (Supplementary Figure S6, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). Additionally, we measured the 42 immune-related proteins in 134 TLS-selected AOs, and compared their expression to the TME AOs. TLSs were defined as spatially organized, non-encapsulated areas of immune cell aggregates. We observed an association between the IGG signature expression and the presence of TLSs in the tumor stroma (Figure 2B). Specifically, 55.2% of tumors with TLSs were IGG-high compared with 25% of tumors without TLSs ($P < 0.001$) (Figure 2B). To better understand the biology associated with TLSs, we focused our attention on the expression of immune proteins in the 134 TLS regions using spatial profiling. Twenty-one proteins (51.2%, 21/41) were found differentially expressed between TLS and non-TLS TME regions (false discovery rate <5%); among them, CD20 (B cell), CD27 (plasma cell), CD11c (dendritic cells), and CD3 (T-cell related) were found more expressed in TLS compared with non-TLS regions, while smooth muscle actin, fibronectin, and CD163 (M2-like macrophages) were found downregulated in TLS compared with non-TLS regions (Supplementary Figure S7A, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). Using the list of the

(D) Distribution of the IGG signature (low, med, high) across age groups in all patients without documented breast cancer recurrence. Chi-square P value <0.001. (E) Forest plot presenting the association of the IGG signature with OS in all patients without documented breast cancer recurrence across different age groups, and in the pooled analysis of the three datasets (in bold). (F) IGG signature expression across breast cancer clinical subtypes in all patients without documented breast cancer recurrence. (G) Distribution of the IGG signature (low, med, high) and age groups, stratified by breast cancer subtype in all patients without documented breast cancer recurrence. CI, confidence interval; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; HR+, hormone receptor-positive; IGG, 14-gene B-cell/immunoglobulin; med, medium; OS, overall survival.

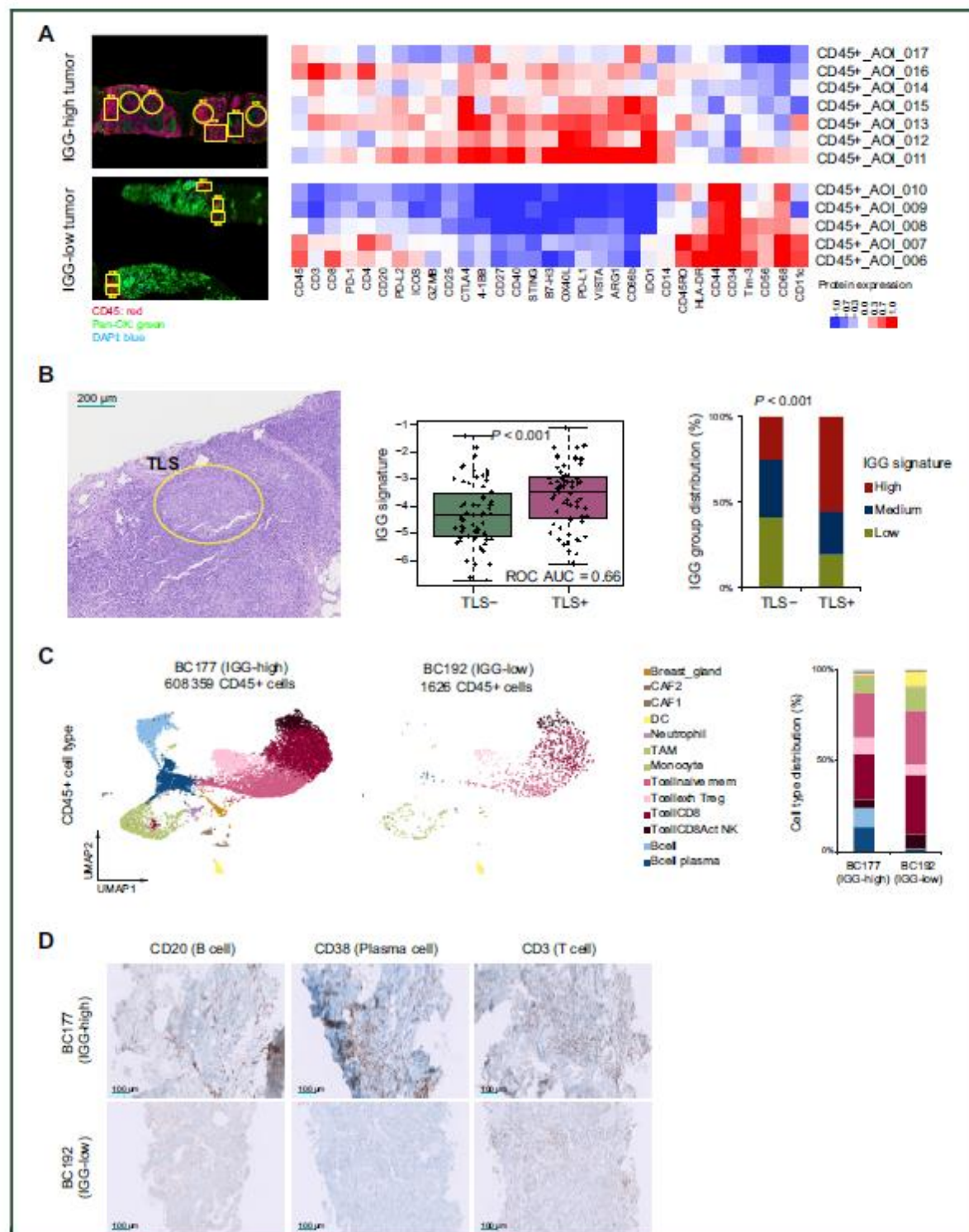


Figure 2. Characterization of the 14-gene IGG signature in breast cancer. (A) Exemplification of protein-based spatial profiling in an IGG-high tumor versus an IGG-low tumor using the GeoMx digital spatial profiling platform. Green denotes tumor cells, red represents immune cells, and blue signifies cell nuclei (DNA). Selected ROIs are highlighted in yellow. The heatmap represents the expression on immune proteins in CD45+ ROIs in each sample. (B) Hematoxylin–eosin staining image depicting a TLS breast tumor (left); IGG signature expression in tumors without TLSs ($n = 69$) versus tumors with TLSs ($n = 66$) (middle); and distribution of IGG signature high, medium, and low expression groups in TLS-negative and TLS-positive tumors (right). (C) UMAP plots showing cell types identified using single-cell RNA sequencing

significant proteins, we established a TLS-related protein signature (Supplementary Data S1, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). We applied this signature to tumor samples that did not visually display TLSs. Notably, we observed a significantly increased expression of this signature in IGG-high tumors compared with those with IGG-medium/low levels (Supplementary Figure S7B, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). This result underscores the IGG signature's capacity to reflect TLS-like biological processes in the tumor stroma, even in the absence of microscopically observable TLSs. Additionally, IGG is significantly associated with TLSs (Supplementary Figure S8, available at <https://doi.org/10.1016/j.esmoop.2024.104109>).

IGG and CD45+ single-cell RNA sequencing

To explore the intricacies of the IGG signature at the cell level, single-cell RNA-seq of fresh CD45+ cells was carried out from six independent breast cancer biopsies with different IGG signature expression levels (Supplementary Figure S9, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). In the tumor with the highest IGG signature score (BC177), 24.0% of CD45+ cells were identified as activated B cells and plasma cells, while these represented only 1.4% of all CD45+ cells in the sample with the lowest IGG signature score (BC192) (Figure 2C). The RNA-seq results observed in these two cases were confirmed by immunohistochemistry staining of CD20 (B cell), CD38 (plasma cell), and CD3 (T cell) (Figure 2D).

Interplay of IGG and B/T-cell clonality

Next, we analyzed BCR and TCR clonality in 23 and 33 breast tumors with known IGG expression values, respectively, using the immunoSEQ DNA-based assay. Overall, a higher polyclonality of B cells and T cells (i.e. lower Simpson clonality index and higher Shannon entropy) was observed in IGG-high tumors compared with IGG-medium/low tumors (Figure 3A and Supplementary Figure S10A, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). Similar results were obtained from an external *in silico* validation breast cancer dataset ($n = 264$, CALGB-40601¹³) based on bulk RNA-seq data (Figure 3B).

Core IGG genes, longevity, and B/T-cell clonality

We then assessed the association of each IGG gene with longevity in the METABRIC, SCAN-B, and TCGA datasets. Seven genes [i.e. *TNFRSF17* (BCMA), *IL2RG*, *POU2AF1*, *CD27*, *IGJ* (JCHAIN), *CD79A*, and *PIM2*], defining the CIGGs, exhibited a statistically significant association with longevity across the three datasets (Figure 3C and Supplementary Table S5, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). A moderate correlation coefficient ($\text{Cor} = 0.63$) among

the CIGGs suggested that they may track slightly different immune components (Figure 3D). To evaluate if the combination of two CIGGs increases the ability to predict longevity over a single CIGG, we added the log base 2 expression values of two CIGGs. The combination of the two CIGGs into a single score provided a better association with longevity, when compared with single CIGG expression ($P < 0.001$) (Figure 3E and Supplementary Table S6, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). Overall, 21 CIGG combinations increased the ability to predict longevity (Supplementary Figure S11A and Supplementary Table S7, available at <https://doi.org/10.1016/j.esmoop.2024.104109>), and the immunoglobulin J chain gene (i.e. *IGJ*, also known as *JCHAIN*) and the interleukin 2 receptor subunit gamma gene (i.e. *IL2RG*) emerged as one of the top gene combinations (Supplementary Figure S11B, available at <https://doi.org/10.1016/j.esmoop.2024.104109>).

Next, we explored the association of each individual CIGG, or any combinations of two CIGGs, with BCR/TCR entropy. The combination of two CIGGs had similar prediction of BCR and TCR Shannon entropy as the IGG signature (Figure 3F and Supplementary Data S2, available at <https://doi.org/10.1016/j.esmoop.2024.104109>). Overall, these results underscore the value of the IGG signature and the combination of CIGGs from bulk RNA to capture an effective adaptive B-cell and T-cell immune response.

IGG and longevity in other cancer types

In the pan-cancer TCGA dataset, we previously reported an association of the IGG signature with OS in cervical cancer ($n = 294$), head and neck cancer ($n = 515$), lung adenocarcinoma ($n = 510$), melanoma ($n = 443$), and sarcoma ($n = 253$).¹⁹ Here we focused on the association of the IGG signature with longevity. Like breast cancer, the association of the IGG signature with OS was found to be independent of cancer relapse in a combined patient-level meta-analysis (hazard ratio 0.68, 95% CI 0.61-0.75, $P < 0.001$) and was consistent across the studied cancer types, except for cervical cancer (Supplementary Figure S12, available at <https://doi.org/10.1016/j.esmoop.2024.104109>).

DISCUSSION

To our knowledge, this study is the first to report an unexpected association between immune gene expression in tumors and longevity in cancer survivors in the absence of recurrence. Notably, high IGG expression, previously linked to reduced distant metastasis and extended survival,^{1,2,4,6} emerges as a reliable marker for extended longevity, signifying its role in sustaining antitumor immunity in breast cancer survivors, and potentially other cancer types. B cells actively contribute to antitumor immunity by producing antibodies and fostering the formation of TLSs, as evidenced by our observed higher B-cell-related gene and

analysis in one IGG-high (BC177) and one IGG-low (BC192) breast cancer sample (left); bar plot distribution of all cell types in each sample (right). (D) IHC staining of CD20 (B-cell marker), CD38 (plasma cell marker), and CD3 (T-cell marker) in one IGG-high (BC177) and one IGG-low (BC192) breast cancer sample. AUC, area under the curve; IGG, 14-gene B-cell/immunoglobulin; IHC, immunohistochemistry; ROC, receiver operating characteristic; ROIs, regions of interest; TLS, tertiary lymphoid structure; UMAP, uniform manifold approximation and projection.

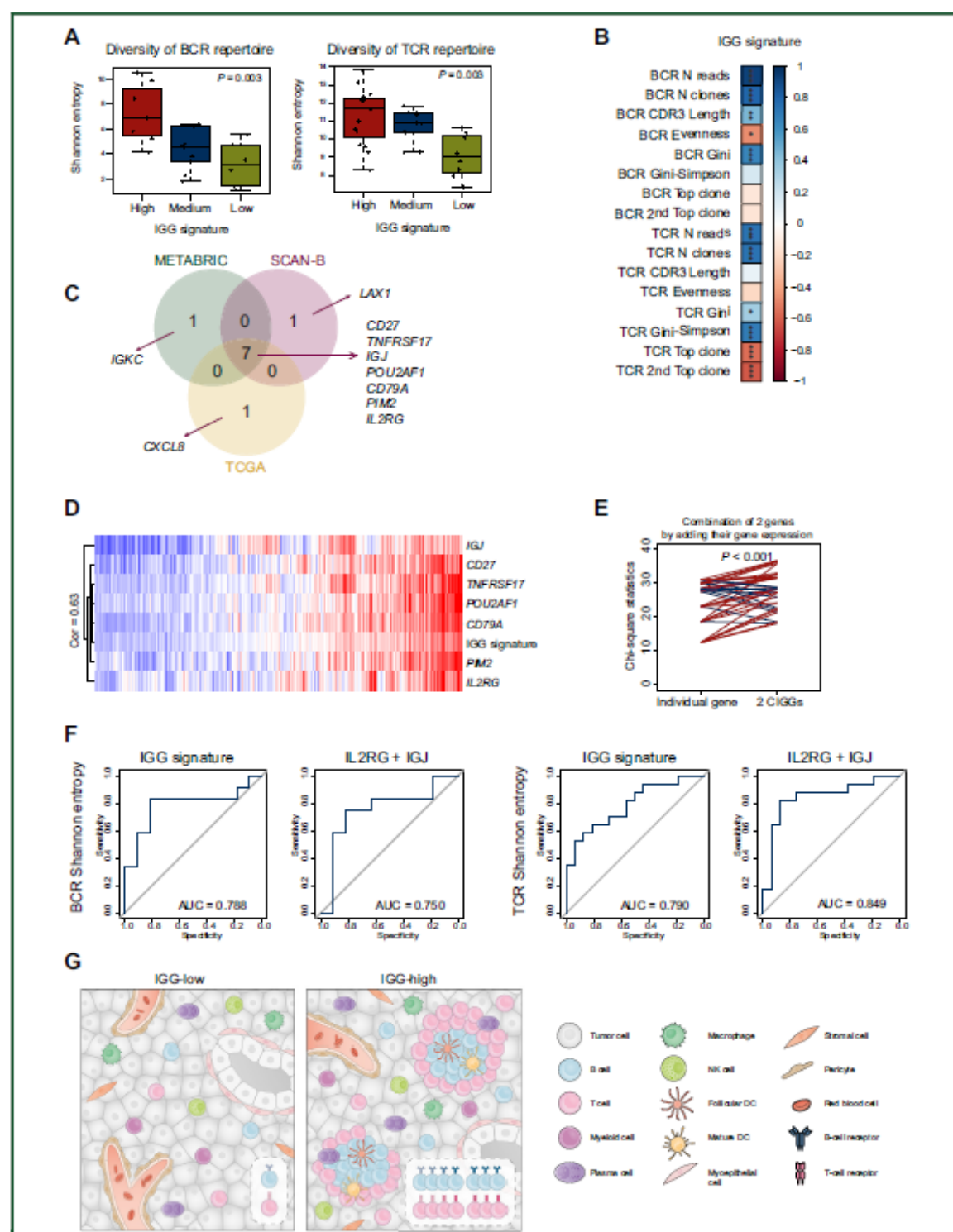


Figure 3. Association between IGG and BCR/TCR clonality and identification of OGGs related to longevity in patients with breast cancer without documented recurrence. (A) BCR Shannon entropy across IGG-high ($n = 10$), IGG-medium ($n = 7$), and IGG-low ($n = 6$) tumors (left) and TCR Shannon entropy across IGG-high ($n = 18$), IGG-medium ($n = 7$), and IGG-low ($n = 8$) tumors assessed by immunoSEQ assay. (B) Pearson correlation between BCR and TCR clonality measures and IGG levels in the HER2-positive breast cancer CALGB-40601 cohort. (C) Venn diagram of the significant individual genes associated with longevity in univariate Cox regression.

protein expression in IGG-high tumors with TLSs. These structures create an environment conducive to B-cell and plasma cell infiltration, promoting a robust and effective adaptive immune response against tumor cells. TLSs play a crucial role in enhancing antitumor immunity and are strongly associated with better clinical outcomes in patients with different solid tumors receiving immune checkpoint inhibitors,²⁰⁻²² suggesting their potential as a biomarker. The identification of TLSs in breast cancer has relied on histology, which only captures a limited two-dimensional section. The IGG signature might offer a more robust method for detecting TLSs, providing a comprehensive view of their presence and dynamics.

The observed polyclonality in B cells and T cells within IGG-high tumors emphasizes the richness and diversity of the immune response, potentially contributing to sustained antitumor immunity and reflecting the robust strength of the patient's immune system. Moreover, this heightened immune vigor suggests increased resilience not only against breast cancer but also against other life-threatening conditions, including other cancer types and infections. A recent study revealed that the composition of B cells in centenarians is shifted from naive to memory cells compared with younger individuals,²³ suggesting a change toward a more robust memory B-cell compartment, which is likely due to their lifetime exposure to a variety of antigens and possibly contributing to their longevity by providing faster and more efficient immune responses.

The identification of CIGGs implicated in various lymphocyte functions further advances our understanding of the molecular determinants of extended survival independently of chronological age. The interaction among the seven identified genes—*TNFRSF17* (BCMA), *IL2RG*, *POU2AF1*, *CD27*, *IGJ* (JCHAIN), *CD79A*, and *PIM2*—constitutes a complex network significantly influencing the longevity of patients with breast cancer. *TNFRSF17* (BCMA), crucial for B-cell maturation and survival, indicates its involvement in sustaining antitumor immunity.²⁴ *IL2RG* contributes to immune response modulation, potentially enhancing the persistence of activated B cells.²⁵ *POU2AF1*, a transcription factor, likely regulates the expression of genes crucial for B-cell function, further supporting the adaptive immune response.²⁶ *CD27*, a member of the tumor necrosis factor receptor superfamily, may contribute to prolonged B-cell activation and antitumor immune memory.²⁷ *IGJ* (JCHAIN) and *CD79A*, essential components of immunoglobulin production, underscore the significance of effective antibody responses in promoting extended survival.^{28,29} Lastly, *PIM2*, a proto-oncogene, may play a role in regulating cell survival and immune cell function.³⁰ The collective action of these genes

highlights the complex molecular mechanisms that contribute to sustained antitumor immunity. Notably, integrating two CIGGs significantly improved predictions of longevity and identified robust T-cell-mediated antitumor responses. Further investigation into their individual and synergistic roles is essential to fully understand their impact on patient outcomes and to guide the development of targeted therapeutic strategies.

Our study has limitations. The observed death events, despite lacking documented recurrence, may potentially be related to undetected recurrences, introducing a degree of uncertainty. Although we focused on patients with a low risk of relapse, the challenge of distinguishing between cancer-related and -unrelated mortality persists. In addition, the cause of death was not available in the datasets analyzed and follow-up time differed across datasets. Nonetheless, the similar observation found in three independent breast cancer datasets, and in other cancer types, decreases the likelihood of a false-positive finding. Finally, our study lacks a detailed mechanistic explanation for the observed associations.

In conclusion, our findings not only contribute to the evolving landscape of prognostic markers in breast cancer but also provide nuanced insights into the relationship between immune gene expression in tumors and longevity, paving the way for more personalized and effective therapeutic strategies that account for both recurrence and long-term outcomes.

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models in METABRIC ($n = 1133$), SCAN-B ($n = 4470$), and TCGA datasets ($n = 936$). (D) Expression of seven CIGGs and IGG signature in the METABRIC dataset. (E) Amount of variation explained in the prognosis of the expression of one single CIGG or the expression of the combination of two CIGGs (i.e. addition of the log base 2 expression values) as defined by chi-square statistics obtained from likelihood ratio testing. (F) ROC AUCs of IGG signature and the combination of the two CIGGs *IL2RG* + *IGJ* to predict BCR Shannon entropy across (left) and TCR Shannon entropy (right). (G) Schematic representation of the characteristics of IGG-low and IGG-high tumors. Spearman correlation: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. AUC, area under the curve; BCR, B-cell receptor; CIGG, core IGG gene; DC, dendritic cell; IGG, 14-gene B-cell/immunoglobulin; NK, natural killer; ROC, receiver operating characteristic; TCR, T-cell receptor.

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DISCLOSURE

Potential conflicts of interest are as follows: LP is an employee of Reveal Genomics and has patents filed: PCT/EP2021/070788, EP23382703, and EP23383369. GV has received a speaker's fee from MSD, Pfizer, GSK, and Pierre Fabre; has held an advisory role with AstraZeneca; and received consultant fees from Reveal Genomics. MMA is an employee of Reveal Genomics. OMS reports advisory/consulting fees from Reveal Genomics, Roche, and AstraZeneca; lecture fees from Daiichi Sankyo, Novartis, Pfizer, and Eisai; and travel expenses from Gilead and Novartis. PG reports part-time employment from Reveal Genomics. FS declares personal fees for educational events and/or materials from Gilead, Daiichi Sankyo, and Novartis; travel expenses from Gilead, Daiichi Sankyo, and Novartis; and advisory fees from Pfizer. HH is a co-founder and shareholder of Omniscope; advisor to NanoString and Mirxes; and consultant to Moderna and Singularity. MG and NB are employees of Omniscope. CMP reports stockholder and consulting fees from Reveal Genomics. AP reports advisory and consulting fees from AstraZeneca, Roche, Pfizer, Novartis, Daiichi Sankyo, and Peptomyc; lecture fees from AstraZeneca, Roche, Novartis, and Daiichi Sankyo; institutional financial interests from AstraZeneca, Novartis, Roche, and Daiichi Sankyo; stockholder and employee of Reveal Genomics; patents filed PCT/EP2016/080056, PCT/EP2022/086493, PCT/EP2023/060810, EP23382703, and EP23383369. FBM reports part-time employment from Reveal Genomics, and has patents filed: PCT/EP2022/086493, PCT/EP2023/060810, EP23382703, and EP23383369. All other authors have declared no conflicts of interest.

DATA SHARING

All genomic and clinical data used in this paper can be retrieved from public repositories. The METABRIC and TCGA data were retrieved from the cBioPortal for Cancer Genomics (<http://cbioportal.org>). Data from the last updated version of the SCAN-B dataset were retrieved from Mendeley Data (<https://data.mendeley.com/datasets/yxxtxn4nmd>). RNA-seq and BCR and TCR repertoire metrics from the CALGB-40601 neoadjuvant HER2-positive study were previously published.¹⁵ The GeoMx raw data generated in this study and the expression of IGG can be found in Supplementary Data S1 and S2, available at <https://doi.org/10.1016/j.esmoop.2024.104109>.

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CLINICAL TRIAL PROTOCOL

ADVANCED-THERAPY MEDICINAL PRODUCT
(ATMP) CLINICAL TRIAL PROTOCOL

A Phase I First-in-Human Dose Escalation Trial to Evaluate the Safety and Efficacy of ARI-HER2 (HER2BBz CAR-T Cells) in Patients with HER2-Positive Advanced Breast Cancer

Protocol code:	ARI-HER2
Study Acronym:	ARI-HER2
EU-CT num.:	2025-521052-35-00
Phase:	I
Sponsor	Fundació de Recerca Clínic Barcelona - Institut d'Investigacions Biomèdiques Agustí Pi i Sunyer (IDIBAPS)
Sponsor' s Acronym:	FCRB-IDIBAPS
Principal Investigator:	Laura Angelats, MD Aleix Prat, MD PhD
Coordinating Investigators	Julio Delgado González

CONFIDENTIAL

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Protocol Number:	ARI-HER2
Version and Date:	Final V1.1, 17 Sep 2025
Investigational Product:	ARI-HER2 cells: Adult autologous differentiated T-cells extracted from peripheral blood, expanded and transduced with a lentivirus to express a chimeric antigen receptor (CAR) targeting human epidermal growth factor 2 (HER2)
EU CT Number:	2025-521052-35-00
Development Phase:	Phase I
Sponsor details:	Fundació Clínic per a la Recerca Biomèdica C/ Villarroel, 170, 08036 Barcelona Phone Number: +34 932 27 57 07
Writing committee:	Written by: Víctor Albarrán Fernández, MD Laura Angelats Company, MD Sara Varea Latorre Elena Guillén Benítez Guillermo Villacampa Javierre, PhD Sara Cano Crespo, PhD Reviewed by: Tomás Pascual Martínez, MD Julio Delgado González, MD Aleix Prat Aparicio, MD PhD Juan Manuel Ferrero-Cafiero, PharmD
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Translational Analysis:	Translational Genomics and Targeted Therapeutics in Solid Tumors Lab (IDIBAPS) / Hospital Clinic Barcelona, Spain
Funding:	AECC

SUMMARY OF THE REVISION HISTORY IN CASE OF AMENDMENTS

Version Nr, Version Date	Chapter	Description of change	Reason for the change

Sponsor and Principal Investigators Signature Page

Title: A Phase I First-in-Human Dose Escalation Trial to Evaluate the Safety and Efficacy of ARI-HER2 (HER2BBz CAR-T Cells) in Patients with HER2- Positive Advanced Breast Cancer

Study ID: ARI-HER2

EU-CT num.: 2025-521052-35-00

The sponsor and the Principal Investigators will conduct the clinical study in compliance with Good Clinical Practice (GCP), with the appropriate regulatory requirements and with the protocol agreed to by the sponsor.

Version: Final V1.1

Study Coordinating Investigator

Julio Delgado González

Signature _____

Date _____

Site Principal Investigators Signature Page**Study ID:**

Title: A Phase I First-in-Human Dose Escalation Trial to Evaluate the Safety and Efficacy of ARI-HER2 (HER2BBz CAR-T Cells) in Patients with HER2-Positive Advanced Breast Cancer
I have thoroughly read and reviewed the trial protocol.

I agree:

- To conduct the trial in accordance with Good Clinical Practice guidelines (GCPs), applicable regulatory requirements and with the approved protocol.
- I confirm that I am thoroughly familiar with the appropriate use of the investigational medicinal product (IMP) as described in the Investigator's brochure and any other information provided by the Sponsor.
- To comply with the procedures for data recording/reporting.
- To ensure that all persons assisting me with the study are adequately informed about the study and of their study-related duties and functions.
- To permit monitoring, auditing and inspection and to retain the trial related essential documents for the period of time required according to ICH-GCP and local regulatory requirements.

Site Principal Investigator

Site name

Name

Signature

Date

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ABBREVIATIONS

ACT: Adoptive Cell Therapy
AE: Adverse Event
AECC: Asociación Española Contra el Cáncer
AESI: Adverse Event of Special Interest
ALT: Alanine Transaminase
AR: Adverse Reaction
AST: Aspartate Transaminase
ASTCT: American Society of Transplantation and Cellular Therapy
ATMP: Advanced-Therapy Medicinal Product
AUX: Auxiliary Medicinal Product
CAR: Chimeric Antigen Receptor
CAR-T: Chimeric Antigen Receptor T-Cell
CBR: Clinical Benefit Rate
CDM: Clinical Data Management
CNS: Central Nervous System
CRA: Clinical Research Associate
CRF: Case Report Form
CRPC: Castration-Resistant Prostate Cancer
CRS: Cytokine Release Syndrome
CTCAE: Classification of Adverse Events
CUN: Clínica Universidad de Navarra, Pamplona
CRO: Contract Research Organization
DLT: Dose-Limiting Toxicity
DOR: Duration of Response
DSMB: Data Safety Monitoring Board
EDC: Electronic Data Capture
ECOG: Eastern Cooperative Oncology Group
EMA: European Medicines Agency
ESA: Erythropoiesis-Stimulating Agents
ETV: Early Termination Visit
FUP: Follow-Up
GCP: Good Clinical Practice
GGT: Gamma-Glutamyl Transferase
GMO: Genetically Modified Organisms
GMP: Good Manufacturing Practice
HAMA: Human Anti-Murine Antibodies
HCB: Hospital Clínic de Barcelona
HDO: Hospital 12 de Octubre, Madrid
ICANS: Immune effector Cell-Associated Neurotoxicity Syndrome
ICF: Informed Consent Form
ICH: International Council for Harmonisation
IEC-HS: Immune Effector Cell-Associated Hemophagocytic
Lymphohistiocytosis-Like Syndrome
IFN- γ : Interferon-Gamma

IL-13R: Interleukin 13 Receptor
IMP: Investigational Medicinal Product
IRB/EC: Ethics Committees for Investigation with Medicinal Products
ISF: Investigator Site File
iRECIST: Immune-based Response Evaluation Criteria in Solid Tumors
ITT: Intent-to-Treat
MAD: Maximum Administered Dose
MHC: Major Histocompatibility Complex
MTD: Maximum Tolerated Dose
NSAID: Nonsteroidal Anti-Inflammatory Drug
ORR: Objective Response Rate
OS: Overall Survival
OTC: Over-the-Counter
PD-1: Programmed Death-1
PI: Principal Investigator
PR: Partial Response
RECIST: Response Evaluation Criteria in Solid Tumors
RP2D: Recommended Phase 2 Dose
RCL: Replication-Competent Lentiviruses
SAEs: Serious Adverse Events
SD: Stable Disease
SUSAR: Suspected Unexpected Serious Adverse Reaction
T-DM1: Trastuzumab Emtansine
T-DXd: Trastuzumab Deruxtecan
TCR: T-Cell Receptors
TEAEs: Treatment-Emergent Adverse Events
TGF- β : Transforming Growth Factor-Beta
TLS: Tumor Lysis Syndrome
TMF: Trial Master File
Treatment-related AEs: Treatment-Related Adverse Events
TYRP1: Tyrosinase Related Protein 1
UAR: Unexpected Adverse Reaction

1. SYNOPSIS

TITLE	A Phase I First-in-Human Dose Escalation Trial to Evaluate the Safety and Efficacy of ARI-HER2 (HER2BBz CAR-T Cells) in Patients with HER2-Positive Advanced Breast Cancer.
PROTOCOL CODE	ARI-HER2
EU-CT NUMBER	2025-521052-35-00
PRINCIPAL INVESTIGATORS	Laura Angelats, MD Aleix Prat, MD PhD
SPONSOR	Fundació Clínic per a la Recerca Biomèdica
FUNDING	Asociación Española Contra el Cáncer (AECC)
CLINICAL RESEARCH ORGANIZATION (CRO)	SOLTI
INDICATION	HER2-positive metastatic or unresectable breast cancer in adult patients who have experienced disease progression or intolerance to standard HER2-targeted therapies.
INTERVENTION	CAR-T cell therapy using HER2BBz CAR-T cells (ARI-HER2)
RATIONALE	Despite advancements in HER2-targeted therapies, a significant proportion of patients develop resistance, leading to an unmet medical need for alternative treatments. CAR-T cell therapy, with its distinct mechanism of action, offers a promising therapeutic strategy for HER2-positive breast cancer patients who have exhausted current treatment options. To date, no studies with HER2-targeted CAR-T cells for the treatment of breast cancer have been published.
STUDY HYPOTHESIS	The administration of ARI-HER2 is safe and well-tolerated in patients with HER2-positive breast cancer, allowing the determination of a recommended dose for future studies.
STUDY DESIGN	This first-in-human, multicenter, open-label, non-randomized Phase I trial follows a 3+3 dose-escalation design to assess the safety and tolerability of ARI-HER2 in patients with HER2-positive metastatic breast cancer refractory to approved treatments.

PRIMARY OBJECTIVES & ENDPOINTS	<table><tr><th>Objectives</th><th>Endpoints</th></tr><tr><td> - To assess the safety and tolerability profile of ARI-HER2 NCI </td><td> - Incidence of grade 3-5 adverse events (AEs) assessed by Common Terminology for Classification of Adverse Events (CTCAE) version 5.0 during the treatment period. - Any grade of AE assessed by CTCAE possibly related to treatment (lymphodepletion chemotherapy and/or HER2CAR) leading to therapy discontinuation. </td></tr><tr><td> - To determine the recommended Phase 2 dose for the expansion phase (RP2D) </td><td> - Dose-limiting toxicity (DLT) defined as the presence of grade 3-5 AEs related to ARI-HER2 assessed by CTCAE v5.0 occurring within 21 days immediately following the last HER2CAR infusion. - Maximum tolerated dose (MTD) defined </td></tr></table>	Objectives	Endpoints	To assess the safety and tolerability profile of ARI-HER2 NCI	- Incidence of grade 3-5 adverse events (AEs) assessed by Common Terminology for Classification of Adverse Events (CTCAE) version 5.0 during the treatment period. - Any grade of AE assessed by CTCAE possibly related to treatment (lymphodepletion chemotherapy and/or HER2CAR) leading to therapy discontinuation.	To determine the recommended Phase 2 dose for the expansion phase (RP2D)	- Dose-limiting toxicity (DLT) defined as the presence of grade 3-5 AEs related to ARI-HER2 assessed by CTCAE v5.0 occurring within 21 days immediately following the last HER2CAR infusion. - Maximum tolerated dose (MTD) defined
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To assess the safety and tolerability profile of ARI-HER2 NCI	- Incidence of grade 3-5 adverse events (AEs) assessed by Common Terminology for Classification of Adverse Events (CTCAE) version 5.0 during the treatment period. - Any grade of AE assessed by CTCAE possibly related to treatment (lymphodepletion chemotherapy and/or HER2CAR) leading to therapy discontinuation.						
To determine the recommended Phase 2 dose for the expansion phase (RP2D)	- Dose-limiting toxicity (DLT) defined as the presence of grade 3-5 AEs related to ARI-HER2 assessed by CTCAE v5.0 occurring within 21 days immediately following the last HER2CAR infusion. - Maximum tolerated dose (MTD) defined						

	as the highest dose

	of HER2CAR that does not cause grade 3-5 AEs (based on CTCAE v5.0)) identified as DLT.
INCLUSION/EXCLUSION CRITERIA	Main inclusion criteria: • >18 years of age • ECOG 0 – 1 • HER2-positive metastatic breast cancer (mBC) • IHC score 3+ in a newly obtained tumor sample • ≥ 2 lines of HER2-directed therapy for mBC (including, or are intolerant to, T-DXd) • At least one measurable and/or evaluable lesion according to RECIST 1.1 criteria. • Life expectancy ≥ 3 months • Last dose of prior anti-cancer therapy at least 28 days (or 5 half-lives). Main exclusion criteria: • Contraindication to receive lymphodepleting chemotherapy • Active infection within 14 days prior to study drug treatment • Systemic therapeutic corticosteroids within 72 hours before CAR-T infusion. • Uncontrolled or significant cardiovascular disease. • Known or suspected leptomeningeal disease or spinal cord compression. • Toxic effects of prior therapies not resolved to CTCAE grade 1 or less (with exceptions). • History or evidence of autoimmune diseases, HIV infection, hepatitis B or C.
SAMPLE SIZE	Included patients: 9-18 patients (minimum of 9, the remaining patients will or will not be enrolled depending on dose-limiting toxicities, see section 12.4).
STUDY MEDICATION, DOSE, AND ROUTE OF ADMINISTRATION	Investigational Medicinal Product (IMP): ARI-HER2 cells: adult autologous differentiated T-cells extracted from peripheral blood, expanded and transduced with a lentivirus containing a low-affinity chimeric antigen receptor anti-HER2 (4D5.5) conjugated with 4-1BB and CD3z Dose: A total dose between 0.15 and 4.5 x10⁶ cell/Kg in 3 administrations (10% of the total on day 0, 30% on day +3 and 60% on day +7) Route of administration: Intravenous (iv)

ESTIMATED ENROLLMENT PERIOD	24 months
ESTIMATED DURATION OF TRIAL	48 months
NUMBER OF SITES	Hospital 12 de Octubre, Madrid (HDO) Clínica Universidad de Navarra, Pamplona (CUN) Hospital Clínic de Barcelona (HCB)

2. GENERAL INFORMATION

2.1 STUDY IDENTIFICATION (ID)

Full title of the trial: A Phase I First-in-Human Dose Escalation Trial to Evaluate the Safety and Efficacy of ARI-HER2 (HER2BBz CAR-T Cells) in Patients with HER2-Positive Advanced Breast Cancer

EU CT Number: 2025-521052-35-00

Protocol Code (or number): ARI-HER2

Study Acronym: ARI-HER2

Version and date: Final V1.1, 17/09/2025

Abbreviated title: Clinical trial to evaluate the safety of CAR T cell ARI-HER2 in the treatment of advanced HER2-positive breast cancer.

2.2 IDENTIFICATION OF THE SPONSOR AND CLINICAL RESEARCH ORGANIZATION

Sponsor:

- Fundació de Recerca Clínic Barcelona - Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) / FCRB-IDIBAPS

Clinical Research Organization (CRO):

- SOLTI, PIC 882423163, established in C/ BALMES 89, 1ER PISO, MODULO 2, BARCELONA 08008, Spain

2.3 IDENTIFICATION OF INVESTIGATORS FROM SPONSOR

Names and affiliations of principal investigators from participating sites are listed in appendix 1 (List of participating sites and principal investigators).

2.4 IDENTIFICATION OF PRINCIPAL INVESTIGATORS OF PARTICIPATING SITES

Names and affiliations of principal investigators from participating sites are listed in appendix 1 (List of participating sites and principal investigators).

2.5 IDENTIFICATION OF THE DECISION MAKER PERSON OR TEAM

Relevant decisions will be agreed by a multidisciplinary group composed of the team of investigators from Hospital Clínic of Barcelona, the production managers of all manufacturing centres and members of clinical pharmacology at Hospital Clínic as pharmacovigilance, regulatory and SOLTI as clinical research organization.

Regarding safety aspects, decisions will be made by a Data Safety Monitoring Board (DSMB) (see section 13.13.1).

2.6 OTHER INSTITUTIONS INVOLVED IN THE TRIAL

Hospital Universitario 12 de Octubre, Madrid (HDO) Clínica Universidad de Navarra, Pamplona (CUN)

3 JUSTIFICATION

3.1 BACKGROUND

Breast cancer is the most common cancer worldwide, accounting for 11.5% of all tumors diagnosed in 2022, and remains one of the leading causes of mortality in women (1). It is a heterogeneous disease encompassing various biological subtypes, including Luminal A, Luminal B, HER2-positive, and triple-negative breast cancer, each with distinct prognoses and oncogenic drivers. Despite significant advancements in surgery, radiotherapy, and systemic treatments such as chemotherapy, endocrine therapy, and targeted therapies, approximately one-third of breast cancer patients will eventually experience relapse and develop metastatic, incurable disease (2).

HER2-positive breast cancer accounts for 15–20% of all breast cancer cases and is characterized by the overexpression and/or amplification of HER2 (human epidermal growth factor receptor 2). This subtype is associated with increased aggressiveness and a poorer prognosis compared to other breast cancer subtypes. However, the introduction of HER2-targeted therapies, including trastuzumab, pertuzumab, trastuzumab emtansine (T-DM1), trastuzumab deruxtecan (T-DXd), and tucatinib, has significantly improved outcomes for patients with HER2-positive metastatic breast cancer. The median overall survival for these patients is now approximately 5 years, with 30–40% surviving 8 years or more (3–5). Despite these advances, over 20% of patients eventually develop resistance to HER2-targeted treatments. Resistance mechanisms associated with trastuzumab and antibody-drug conjugates (ADCs) such as T-DM1 and T-DXd include alterations in HER2 (e.g., loss of expression or mutations), changes in proteins involved in the HER2 signaling pathway, impaired internalization of the HER2-drug complex, lysosomal degradation, and chemotherapy resistance linked to these ADCs. This subset of patients represents a significant unmet medical need, necessitating the development of novel treatment strategies (6–8).

Chimeric antigen receptor (CAR) T-cell therapy offers a unique mechanism of action distinct from trastuzumab, T-DM1, and T-DXd. Following activation, CAR T-cells can induce the destruction of target cells through various apoptosis-inducing mechanisms, including osmotic lysis (mediated by perforin and granzyme B) and FAS-FASL interaction (9,10). Therefore, CAR-T cell therapy could represent a promising therapeutic alternative for patients with alterations in internalization, lysosomal degradation, and/or resistance to chemotherapy.

3.1.1 CAR-T cell therapy

Immunotherapy has revolutionized the treatment landscape for both solid and hematologic tumors. Among various immunotherapy approaches, adoptive cell therapy (ACT) has garnered significant attention over the past decade. ACT is a personalized treatment in which a patient's immune cells are isolated, expanded in vitro, and subsequently reinfused to target and eliminate tumors.

The earliest ACT strategy involved tumor-infiltrating lymphocytes (TILs) extracted from the tumor tissue of melanoma patients. Subsequently, advances in genetic engineering enabled the development of T cells modified with synthetic receptors, such as T-cell receptors (TCRs) or chimeric antigen receptors (CARs). CARs are hybrid receptors engineered to include an extracellular domain (derived from the single-chain variable fragment [scFv] of an antibody), intracellular signaling domains, and co-stimulatory domains that collectively induce T-cell activation. By introducing a CAR into a T cell, the cell is reprogrammed with a new, tumor-specific antigen recognition capability (11,12).

CAR-T therapy has demonstrated remarkable clinical responses in patients with

hematologic malignancies, including sustained complete remissions (13). Since 2017, seven commercial CAR-T cell products have been approved in the United States (six in the European Union) for the treatment of B-cell malignancies. In contrast to hematologic tumors, solid tumors present additional layers of complexity. T cells must first navigate through the circulatory system, reach the tumor site, and successfully infiltrate the tumor. Once there, they must withstand an immunosuppressive tumor microenvironment while maintaining their effector functions. Additionally, T cells face the challenge of detecting tumor cells amidst tumor heterogeneity. Consequently, leveraging CAR-T cells for the treatment of solid tumors represents the next significant challenge and opportunity in this field (14, 15).

Clinical trials involving patients with solid tumors suggest that CAR-T cells are capable of infiltrating tumors and exhibiting antigen-specific activity. Some indications of clinical efficacy have been observed; however, most patients do not respond to CAR-T cell therapy, experience early relapse, or develop toxicities. In the context of solid tumors, the primary adverse events have been 'on-target off-tumor' toxicities, which occur when healthy tissues that express low levels of the target antigen are inadvertently damaged (16). The most straightforward strategy to avoid toxicities is to target antigens that are exclusively expressed on tumor cells, but not on normal tissue. However, identifying suitable antigens for solid tumors has been challenging due to their shared expression in healthy tissues. An alternative approach is to adjust the affinity of the antigen recognition domain, allowing it to distinguish between tumor and normal tissues based on differing levels of antigen expression (15).

In one of the first clinical trials reported, treatment with a high dose (10^{10} cells) of high-affinity CAR-T cells targeting HER2 resulted in lethal toxicity due to T-cell accumulation in the lungs (17). However, more recent studies have shown that CAR-T cells targeting HER2, using a different scFv, are safe and demonstrate promising antitumor activity in some sarcoma patients, including a complete response in a pediatric patient (18,19).

Interim results from a recent phase I trial involving Claudin18.2 (CLDN18.2)-targeted CAR-T cells have shown promising efficacy in heavily pre-treated patients with CLDN18.2-positive gastrointestinal cancers. The trial reported an overall response rate (ORR) of 48.6% and a disease-control rate (DCR) of 73.0%, with no grade ≥ 3 cytokine release syndrome (CRS) or signs of neurotoxicity (20). Claudin 6 (CLDN6) represents another promising target in solid tumors. An ongoing phase 1/2 trial combining CLDN6-targeted CAR-T therapy with an amplifying RNA-based vaccine (BNT211-01) has reported an overall response rate (ORR) of 33% and a disease-control rate (DCR) of 67% in 21 evaluable patients, including one complete response, with manageable toxicity. (21).

CAR-T cells targeting prostate-specific membrane antigen, transduced with a negative transforming growth factor (TGF)- β receptor, have been evaluated in patients with castration-resistant prostate cancer (CRPC). The results were modest, with stable disease observed in 5 out of 13 patients, and the therapy exhibited an overall good safety profile (22).

GD2-targeted CAR-T cell therapy has shown promising results in pediatric patients with relapsed or refractory high-risk neuroblastoma (ORR 63%, including 9 complete responses among 27 patients) (23). Another study showed the feasibility and early signs of efficacy of regionally delivered GD2-CAR-T cells in patients with H3K27M-mutated pediatric diffuse midline glioma (24). The interleukin 13 receptor (IL-13R) has been identified as a promising target in several solid tumors (25); the intrathecal administration of IL-13R $\alpha 2$ -targeting CAR-T cells has shown efficacy in patients with recurrent high-grade glioma (26), and there is an ongoing phase I clinical trial in patients with advanced melanoma (NCT03119024).

In malignant pleural mesothelioma, a phase I trial reported promising results for the intrapleural administration of mesothelin-targeted CAR-T therapy in combination with pembrolizumab, supported by preclinical data suggesting that PD-1 blockade enhances CAR-T cell function (27). Tyrosinase-related protein 1 (TYRP1) has recently emerged as a promising target for CAR-T cell therapy in both cutaneous and non-cutaneous melanoma, showing encouraging preliminary efficacy in patient-derived murine models (28).

To date, no studies have been published or are in development for the use of HER2-targeted CAR-T cells in breast cancer treatment. However, there are eighteen ongoing Phase 1 clinical trials, all based in the United States and China. Five of these focus on HER2-positive brain tumors or metastases (NCT03696030, NCT03500991, NCT05768880 and NCT02442297 evaluate locoregional delivery of anti-HER2 T cells, while NCT03383978 examines the delivery of anti-HER2 NK cells). The remaining trials assess intravenous administration of different immune cells transduced with anti-HER2 CARs in combination or in monotherapy, for an array of HER2 overexpressing solid tumors: NCT04660929 (CAR-macrophages), NCT03740256 (CAR-T cells in combination with an oncolytic virus), NCT04995003 (CAR-T cells combined with PD-1 inhibitors), NCT04684459 (dual CARs targeting HER2 and PD-L1), NCT06251544 (dual TRAIL-R2 and HER2 bispecific CAR-T cells), NCT04430595 (multiple 4th generation CARs), and NCT06241456 (CAR-T cells combined with anti-EGFR monoclonal antibodies). Several trials (NCT04511871, NCT06101082, NCT05745454, NCT03198052, NCT04903080, and NCT01109095) are exploring HER2-targeted CAR-T cells as monotherapies for various solid tumors distinct from breast cancer. Notably, the first three investigate anti-HER2 CAR-T cells for broad range of solid tumors, NCT03198052 investigates also other antigens (GP3/mesothelin/Claudin18.2) as CAR targets, NCT04903080 focuses on pediatric ependymoma, and NCT01109095 on adult patients with glioblastoma.

3.1.2 Justification of anti HER2-CAR T cells (ARI HER2)

The research team at Hospital Clínic de Barcelona has developed a CAR that recognizes HER2 with low affinity, designed for potential clinical use in the treatment of HER2+ metastatic breast cancer (ARI-HER2). ARI-HER2 consists of autologous human T cells genetically modified to specifically target any human cell that overexpresses HER2 on its surface, including malignant cells from HER2-positive breast cancer patients.

ARI-HER2 cells are generated by transducing T cells with a lentiviral vector that expresses a CAR containing a low-affinity 4D5.5 single-chain variable fragment (scFv). This scFv is derived from trastuzumab but has been modified with two-point mutations to reduce its affinity. ARI-HER2 incorporates the CD8 transmembrane domain and the 4-1BB intracellular domain.

ARI-HER2 cells are adult autologous differentiated T cells, extracted from peripheral blood, expanded, and transduced with a lentivirus encoding the anti-HER2 chimeric antigen receptor (CAR) conjugated with 4-1BB and CD3ζ. The rationale behind the development of ARI-HER2 cells for treating HER2-positive breast cancer lies in the overexpression of the HER2 antigen in this breast cancer subtype.

To assess the efficacy and safety of ARI-HER2, we compared our CAR with one that has previously been associated with toxicity issues in clinical trials. For this, we selected a third-generation CAR developed in the early 2000s at the NIH, which recognizes HER2 with high affinity (trastuzumab-derived scFv, 4D5). Apart from the scFv, the NIH-developed CAR (HER2-28BBz HA NIH) differs from ARI-HER2 (HER2-BBz LA) in the leader sequence, hinge and transmembrane domains, and intracellular domains.

In preclinical assays, we compared the therapeutic index of our low-affinity CAR with the NIH-developed CAR in models expressing various HER2 densities, as well as in a panel of normal cells. Additionally, we evaluated the efficacy and toxicity of ARI-HER2 in in vivo mouse models, comparing it to the NIH CAR (HER2-28BBz).

For further details on the non-clinical assays, please refer to the Investigator's Brochure.

3.2 SUMMARY OF THE KNOWN AND POTENTIAL RISKS AND BENEFITS

3.2.1 Known and potential risks

Adverse effects can be categorized into those directly associated with the infusion of ARI-HER2 cells and those arising from the overall treatment process.

Foreseeable adverse effects directly related to the infusion of ARI-HER2 cells:

According to EMA guidelines, an important risk is defined as a security risk that requires specific and specialized management to investigate and minimize the risk.

Within this category, the following distinctions are made:

- **Important identified risks**, which are the risks identified in the clinical development plan and associated with sufficient evidence for each CART product.
- **Important potential risks**, which are risks for which prior published evidence is available, but which have not been identified for the time being in the clinical development plan or which have been reported, but without robust evidence that they can be associated to the CART product.

Based on the available literature and insights gained from CAR-T cell products developed at HCB, the following risks have been postulated for ARI-HER2:

Important risk	
Identified risks	• Cytokine release syndrome (CRS)/IEC-HS (Immune Effector Cell- Associated Hemophagocytic Lymphohistiocytosis-Like Syndrome). • Immune effector Cell-Associated Neurotoxicity Syndrome (ICANS), including serious manifestations as brain oedema and parkinsonism. • Febrile neutropenia and infections. • Persistent cytopenias.
Potential risks	• Tumor lysis syndrome (TLS). • Secondary malignancies. • Immunogenicity/Autoimmunity. • Transmission of adventitious agents. • Infusion reactions. • Generation of replication-competent lentiviruses (RCL). • Decreased cell viability due to improper product handling. • On-target off-tumor toxicity derived from the damage of HER2-expressing healthy tissues (i.e., interstitial lung disease/pneumonitis, decrease in ventricular ejection fraction, diarrhea/gastrointestinal toxicity, alteration of liver enzymes).

Table 1. Risks associated with ARI-HER2 therapy.

Other risks associated with apheresis and lymphodepleting chemotherapy are outlined below, categorized by their frequency:

Local effects in the venous puncture area for apheresis and blood extractions	▪ <i>Frequent</i>: Local bruising, bleeding, irritation and discomfort in the venous puncture area, bacteremia from the same puncture without serious consequences. ▪ <i>Uncommon</i>: Apheresis catheter sepsis (serious), venous line related infection, local allergic reaction to venous line material.
Haematological system	▪ <i>Very common</i>: Neutropenia, thrombocytopenia, and lymphopenia directly related to lymphodepleting chemotherapy (potentially serious). ▪ <i>Common</i>: Anemia directly related to apheresis (potentially serious). ▪ <i>Uncommon</i>: Apheresis-related thrombocytopenia (potentially severe), eosinophilia if there is any type of

	allergic reaction.
Nervous system	▪ <i>Common</i>: Dizziness or vertiginous feeling, paresthesia in the venous puncture site or generalized, headache. ▪ <i>Uncommon</i>: Cerebrovascular accident (severe), hypoesthesia, tremor.
Cardiovascular system	▪ <i>Common</i>: Dizziness, hypotension or hypertension (potentially serious if uncontrolled), non-specific chest pain. ▪ <i>Uncommon</i>: Syncope (risk of seriousness), atrial fibrillation (serious), myocardial infarction (serious), symptoms of heart congestive failure.
Respiratory system	▪ <i>Common</i>: Non-specific dyspnea, nonspecific chest pain. ▪ <i>Uncommon</i>: Bronchospasm (severe), hypoxia (serious).
Gastrointestinal system	<i>Very common</i>: elevation of AST, ALT, GGT, alkaline phosphatase, and bilirubin, directly related to lymphodepleting chemotherapy (potentially serious), especially in patients with a history of hepatotoxicity due to prior chemotherapy treatments. ▪ <i>Common</i>: non-specific abdominal pain, nausea or vomiting. ▪ <i>Uncommon</i>: intestinal rhythm disturbance.
Skin and subcutaneous tissue	▪ <i>Common</i>: pruritus, hyperhidrosis, urticaria (potentially severe), hematoma and local reaction of the venipuncture site. ▪ <i>Uncommon</i>: generalized rash due to allergic reaction (severe).
Musculoskeletal system	▪ <i>Common</i>: Myalgia and arthralgia, spasms or muscle contractures ▪ <i>Uncommon</i>: Arthritis (severe).
Genito-urinary system	▪ <i>Frequent</i>: Non-specific discomfort when urinating. ▪ <i>Uncommon</i>: Hematuria (potentially severe), renal colic (serious).
General adverse effects	▪ <i>Common</i>: Asthenia, fatigue, fever, dizziness, tetany, generalized ▪ pain, flu-like symptoms

Table 2. Apheresis and lymphodepleting chemotherapy-associated risks categorized by their frequency: very common (>10%), common (1-10%), frequent (1-10%), uncommon (0.1-1%).

3.2.2 Known and potential benefits

CAR-T cell therapy has demonstrated the potential to induce deep and long-lasting clinical responses in hematologic malignancies, particularly those with homogeneous expression of targetable antigens resulting from oncogene alterations with critical roles in tumorigenesis. In contrast, HER2 overexpression in HER2-positive breast tumors represents a well-established example of oncogene addiction, providing a strong rationale for the application of CAR-T cell therapy.

Even in patients who achieve deep responses to anti-HER2 monoclonal antibodies or antibody-drug conjugates (ADCs), clinical benefit is often eventually lost. This acquired resistance is typically due to biological changes in tumor cells that promote receptor-drug complex degradation or internalization, as well as the emergence of mutations in downstream HER2-related oncogenic signaling pathways. However, HER2-CAR T cells

can induce necrosis and/or apoptosis in malignant cells upon antigen recognition, regardless of HER2 functional status or the presence of downstream genetic alterations. Therefore, HER2-CAR T cells are expected to remain effective against HER2-positive breast cancer despite these resistance mechanisms.

As a low-affinity HER2-CAR-T therapy, ARI-HER2 is specifically designed to induce toxicity primarily in HER2-positive cells, sparing HER2-low cells such as normal epithelial cells in healthy tissues. While this limits ARI-HER2's activity against HER2-low breast cancer, it is expected to significantly reduce or avoid on-target off-tumor toxicity in patients with HER2-positive tumors, a major concern associated with previous high-affinity HER2-targeted CAR-T cell therapies.

Patients with advanced HER2-positive breast cancer who progress on anti-HER2 antibodies and ADCs (such as TDM-1 and T-DXd) currently lack effective treatment options beyond conventional chemotherapy, which rarely offers long-term clinical benefit. While acute toxicities related to lymphodepleting chemotherapy and CAR-T cell infusion, particularly cytokine release syndrome (CRS), are anticipated, ARI-HER2 has the potential to address this critical medical need in a significant subgroup of patients. Like other immunotherapy modalities, including adoptive cell therapies for both hematologic and solid malignancies, ARI-HER2 could potentially mediate sustained clinical responses in certain patients. Given that the anticipated adverse events are expected to be short-term and manageable, and considering that ARI-HER2 is a one-time treatment, its long-term impact on the quality of life for these patients is expected to exceed that of conventional therapies.

3.3 INVESTIGATIONAL PRODUCT

ARI-HER2 cells are adult autologous differentiated T-cells extracted from peripheral blood, expanded and transduced with a lentivirus.

Participants will undergo lymphoapheresis to collect T-cells from peripheral blood (50–250 mL). Before apheresis, a CD3+ T-cell count of at least 100 cells/ μ L and negative serologies are required. Each apheresis product must contain at least 400 million CD3+ T-lymphocytes, or 800 million in the case of cryopreserved apheresis. For detailed logistical and handling requirements, please refer to the Sample Management Guide.

ARI-HER2 cells, will be manufactured in a commercial bioreactor (CliniMACS Prodigy, Miltenyi) following a process like that used for other HCB products (29,30)

The infusion product will be packaged in three 30 mL bags, with the total dose divided into three aliquots, each corresponding to the patient's cohort and weight. ARI-HER2 cells will be administered intravenously, with fractioned dosing based on our previous experience with ARI-0001 and ARI0002h cells. We plan to administer between 0.15 and 4.5×10^6 ARI-HER2 cells/kg in 3 fractions, as described in section 6.2. For detailed logistical and handling requirements, please refer to the Sample Management Guide.

3.3.1 Mechanism of action

ARI-HER2 is an autologous human T-cell product genetically modified to express a low-affinity chimeric antigen receptor (CAR) that targets HER2. This design allows for specific binding to human cells that overexpress HER2 receptors on their surface, such as HER2-positive breast cancer cells, while minimizing significant interactions with HER2-low expressing cells, like those found in healthy epithelial tissues. This reduces the risk of on-target off-tumor toxicity.

Upon engagement with HER2-positive malignant cells, HER2-CAR T cells induce tumor cell killing in an MHC-independent manner. This occurs through several mechanisms, including:

- The production of perforins and granzymes

- Induction of apoptosis via the FAS/FAS ligand axis
- The release of immune-stimulating cytokines to sensitize the tumor microenvironment.

Our preclinical data show that the recognition of HER2 receptors by ARI-HER2 triggers the production of high levels of IFN- γ and IL-2, leading to cytotoxic effects against HER2-positive cancer cells.

3.3.2 Dose justification

To the best of our knowledge, only one clinical trial and two case reports have been published involving CAR-T therapy targeting HER2. To determine the appropriate dose escalation for our study, the research team reviewed previously published Phase 1 clinical trials involving CAR-T therapy in solid tumors (Table 3) (17–24,26,27,31–36). These trials show a variable dose range of 1×10^6 - 1×10^8 cells/m², 1×10^5 - 1×10^8 cells/kg or 2.5×10^8 - 1×10^{10} total cells. No severe adverse events were observed in these trials, except for the first study published in 2010 with a CAR against HER2 that administered 10^{10} total cells and resulted in a patient fatality.

Recently, Qi C et al. (20) demonstrated the efficacy of a CAR-T therapy targeting Claudin 18.2 in patients with previously treated metastatic digestive cancer. During the expansion phase of the trial, a dose of 2.5×10^8 total cells was used, showing good tolerance and achieving an objective response rate (ORR) of 48.6%. Based on the doses, efficacy, and adverse events of the clinical trials cited above, the dose escalation selected for our clinical trial is 1.5 to 4.5×10^6 CAR-T cells/kg intravenously.

Target	Co-stimulation domain	Dose	Tumor Type	Adverse events	Reference
Anti-HER2 (n=1)	CD28 and 4-1BB	10^{10} cells	Colorectal cancer	Death	Morgan et al. Mol Ther 2010
Anti-HER2 (n=1)	CD28	1×10^8 cells/m ² x 2 cycles	Rhabdomyosarcoma	CRS G1	Hedge et al., Nat Commun 2020
Anti-HER2 (n=17)	CD28	1×10^6 - 1×10^8 cells/m ²	Glioblastoma	Seizures and headache G2	Ahmed N et al., JAMA Oncol 2017
Anti-mesothelin (n=27)	CD28	3×10^5 - 6×10^7 CAR T cells/Kg	Mesothelioma, NSCLC, breast cancer	CRS G<2	Adusumilli PS et al., Cancer Discov 2021
Anti-mesothelin (n=15)	CD28	0.18 - 11×10^6 CAR T cells/Kg	Solid tumors (MSLN+)	Ascites and pericardial/pleural effusion G1-G3	Wang Z et al., Cell Mol Immunol 2021

Anti-claudin 18.2 (n=37)	CD28	2.5x10 ⁸ – 5x10 ⁸ cells	Gastro-esophageal / pancreatic cancer	CRS G1-G2	<i>Qi C et al. Nat Med, 2022</i>
Anti-CEA (n=10)	CD28	1x10 ⁵ - 1x10 ⁸ cells/Kg	Colorectal cancer	None	<i>Zhang C et al, Molecular therapy 2017</i>
Anti-EGFR (n=11)	4-1BB	0,04x10 ⁷ – 2,54x10 ⁷ cells/Kg	NSCLC	Hyperlipasemia G3-4, rash G1-2, dyspnea G1-2	<i>Feng K et al, sci chi 2016</i>
Anti-PSMA (n=13)	CD28	1x10 ⁷ - 3x10 ⁸ cells/m2	mCRPC	CRS G3-G4 Death (1 patient)	<i>Narayan V et al, Nat Med 2022</i>
Anti-claudin 6 (n=22)	4-1BB	1x10 ⁷ - 1x10 ⁸ cells/Kg	Solid tumors (CLDN6+)	CRS G3-G4	<i>Mackensen et al, Nat Med 2023</i>
Anti-cMET (n=7)	CD28	1x10 ⁸ cells	Melanoma, TNBC	CRS G1-G2	<i>Shah P et al, Cancer Res Commun 2023</i>
Anti-GPC3 (n=6)	4-1BB	250x10 ⁶ cells	Hepatocellular carcinoma	CRS G3	<i>Fu Q et al, Clinical Medicine 2023</i>
Anti-GD2 (n=4)	4-1BB	1x10 ⁶ cells	Diffuse midline gliomas	CRS G1-G2	<i>Majzner R et al, Nature 2022</i>
Anti-GD2 (n=27)	CD28	3-10x10 ⁶ /kg cells	Neuroblastoma	CRS G1-G2	<i>Del Bufalo F et al, NEJM 2023</i>
Anti-EGFR/IL13R (n=6)	CD28	1-2.5x10 ⁷ cells	Glioblastoma	ICANS G3 CRS G1-G2	<i>Bagley S et al, Nat Med 2024</i>
Anti-IL13R (n=65)	4-1BB	10-200x10 ⁶ cells	High grade glioma	ICANS G3	<i>Brown C et al, Nat Med 2024</i>

Table 3. Published phase I trials with CAR-T therapies for patients with solid tumors. CRS: cytokine- release syndrome; ICANS: immune effector cell-associated neurotoxicity syndrome; NSCLC: non- small cell lung cancer; mCRPC: metastatic castration-resistant prostate cancer; TNBC: triple- negative breast cancer; GPC3: glypican 3.

To minimize the risk of on-target off-tumor toxicity and cytokine release syndrome, the dose will be fractionated and administered in 3 doses over 7 days: 10% of the total on day 0, 30% on day +3 and 60% on day +7.

Additionally, as a risk minimisation measure and to evaluate potential toxicities, the study will follow a dose-escalation 3+3 design, subdivided into 3 cohorts.

Three cohorts have been defined (tabulated in Table 6, section 7.2):

- The first cohort would receive between $0.15\text{--}1.5 \times 10^6$ ARI-HER2 cells/Kg in three fractions corresponding to 10, 30 and 60% respectively (0.15 , 0.45 and 0.90×10^6 ARI-HER2cell/Kg).
- The second cohort would receive between $0.30\text{--}3 \times 10^6$ ARI-HER2 cells/Kg in three fractions corresponding to 10, 30 and 60% respectively (0.30 , 0.90 and 1.8×10^6 ARI-HER2cell/Kg).
- The third cohort would receive between $0.45\text{--}4.5 \times 10^6$ ARI-HER2 cells/Kg in three fractions corresponding to 10, 30 and 60% respectively (0.45 , 1.35 and 2.7×10^6 ARI-HER2cell/Kg).

A minimum follow-up of 28 days will be required after the administration of the last dose fraction of each patient before treating the next patient.

4 TRIAL OBJECTIVES AND ENDPOINTS

4.1 CO-PRIMARY OBJECTIVES AND ENDPOINTS

Co-primary objectives	Endpoints
To assess the safety and tolerability profile of ARI-HER2	• Incidence of grade 3-5 adverse events (AEs) assessed by NCI Common Terminology for Classification of Adverse Events (CTCAE) version 5.0 during the treatment period. • Any grade of AE assessed by CTCAE possibly related to treatment (lymphodepletion chemotherapy and/or HER2CAR) leading to therapy discontinuation.
To determine the recommended phase 2 dose for the expansion phase (RP2D).	• Dose-limiting toxicity (DLT) defined as the presence of grade 3-5 AEs related to ARI-HER2 assessed by CTCAE v5.0 occurring within 21 days immediately following the last HER2CAR infusion. • Maximum tolerated dose (MTD) defined as the highest dose of HER2CAR that does not cause grade 3-5 AEs (based on CTCAE v5.0) identified as DLT.

4.2 SECONDARY OBJECTIVES AND ENDPOINTS

Secondary objectives	Endpoints
To evaluate the efficacy of ARI-HER2 in patients with R/R HER-2 positive metastatic breast cancer	• Clinical Benefit Rate (CBR) defined as the percentage of patients who have a complete response (CR), partial response (PR) or stable disease (SD) lasting >24 weeks, based on local investigator's assessment according to iRECIST criteria (see section 8.1 for additional details). • Objective response rate (ORR): locally determined as the best response (defined as CR and PR) by the investigator according to RECIST 1.1 criteria after treatment administration (see section 8.1 for additional details). • Duration of response (DOR) defined as the time from the first occurrence of a documented objective response to disease progression as determined locally by the investigator using RECIST v1.1 or death (whichever occurs first). See section 8.1 for additional details. • Progression-free survival (PFS) defined as the time from administration of the ARI-HER2 product to disease progression or death (whichever is earlier). See section 8.1 for additional details. • Overall survival (OS) defined as the time from administration of the ARI-HER2 product to death from any cause (see section 8.1 for additional details).
To assess the treatment compliance of ARI-HER2	• All changes in treatment administration including delays, interruptions, reductions or discontinuation and the main reason(s) for these changes.
To evaluate the persistence of ARI-HER2 in blood.	• CAR persistence defined as percentage of HER2CAR in the blood weekly during the first month, monthly until the first year and every three months until 2 years of follow-up.
To evaluate the incidence, severity and duration of cytokine release syndrome and neurotoxicity syndrome in subjects after ARI-HER2 administration.	• Cytokine induction defined as the type and number of cytokines released into the blood following HER2CAR infusion, as well as at which time point it occurs. • Incidence and grade of cytokine release syndrome (CRS) and Immune effector Cell-Associated Neurotoxicity Syndrome (ICANS) from ARI-HER2 infusion and during follow-up.
To assess middle and long-term toxicity	• Incidence of grade 3-5 adverse events (AEs) assessed by NCI Common Terminology for Classification of Adverse Events (CTCAE) version 5.0 at months 3, 6 and 12 after ARI- HER2 infusion.

To evaluate the association of the HER2DX genomic test with clinical outcomes	Correlation between the expression of gene signatures with validated prognostic value in HER2+ BC assessed by HER2DX genomic test with efficacy and survival outcomes (CBR, ORR, DOR, PFS, OS).

4.3 EXPLORATORY OBJECTIVE

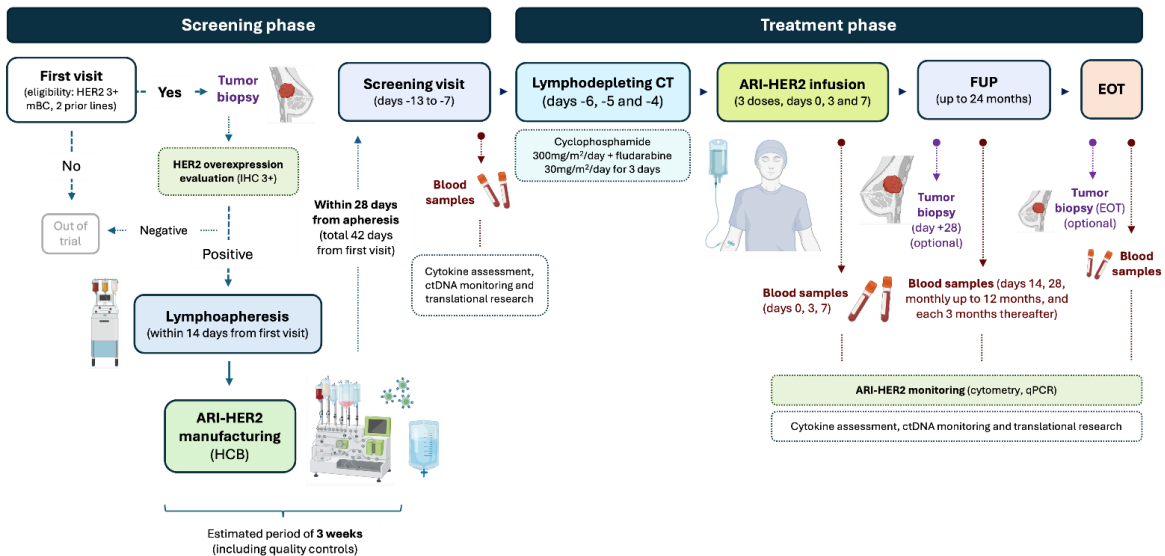
The exploratory objective of this study is to evaluate translational data from biopsies and blood samples obtained during the trial. Various research studies will be conducted in the future as separate investigations, including immunophenotyping, immunohistochemistry, RNA sequencing, gene expression and DNA sequencing. These samples will be stored in a biobank until the initiation of these studies.

5 STUDY DESIGN

5.1 OVERVIEW OF CLINICAL TRIAL DESIGN

This study is a first-in-human, multicenter, open-label, prospective, non-randomized, dose-escalation clinical trial using a 3+3 design to assess the safety and tolerability of ARI-HER2 in patients with HER2-positive metastatic breast cancer who are refractory to approved treatments. The trial design is summarized in Figure 1.

Figure 1. Study design



Patients will be enrolled in the study in strict sequential order. The administration of ARI-HER2 cells will be staggered. Each patient will be monitored for a minimum of 28 days after the first fraction of ARI-HER2 (corresponding to the complete DLT assessment period) before the next patient in the same cohort can initiate treatment. This sequential dosing rule applies across all sites and will be overseen centrally by the Medical Monitor.

The study will consist of five distinct phases:

- Patient Screening:** After obtaining informed consent, a fresh tumor biopsy will be performed to confirm HER2 status. If the results meet the selection criteria, as validated by the medical monitor, a leukapheresis procedure will be conducted to collect sufficient

peripheral mononuclear cells.

- **Preparation of the Investigational Drug:** ARI-HER2 cells will undergo a manufacturing process lasting 7–10 days, followed by a two-week period for quality control evaluation.
- **Patient Inclusion:** Once successful production of the investigational product is confirmed, the inclusion and exclusion criteria will be reviewed during the screening visit. Upon approval by the medical monitor, the patient will be officially included in the study.
- **Treatment Period:** This phase will involve lymphodepleting chemotherapy, followed by a fractionated infusion of ARI-HER2 cells. Pre- and post-medication will be administered as detailed in Section 2.3.2.
- **Follow-up:** Patients will be monitored for at least 24 months following the first ARI-HER2 infusion. Additionally, in accordance with good clinical practices for advanced therapies, long-term safety follow-up will be conducted for up to 15 years post-infusion, in collaboration with the Hospital Clinic of Barcelona, the holder of ARI-HER2.

Different study populations will be defined according to the following criteria:

- P0: patients who sign the ICF.
- P1: patients who meet the inclusion criteria and undergo lymphoapheresis.
- P2: patients in whom successful manufacturing of the cell product is achieved and the screening phase is completed.
- P3: patients who proceed to the treatment phase and receive lymphodepletion chemotherapy.
- P4: patients who receive at least 1 fraction of the cell product (ARI-HER2).
- P5: patients receiving all 3 fractions of the cell product (ARI-HER2).
- P6: patients receiving any fraction of the cell product (ARI-HER2) in which no major protocol deviations have been made.

These populations will be considered for the statistical analysis, taking P3 as the reference population for the safety and P4 for the efficacy assessment.

5.2 MINIMIZATION/AVOIDANCE OF BIAS

There will be no randomization, control, or blinding as this is a pilot study with a single arm. Patients who meet all inclusion criteria and none of the exclusion criteria will be eligible for inclusion. Those who consent to participate will be treated with ARI-HER2, unless a condition arises that makes treatment infeasible (e.g., disease progression).

5.3 DURATION OF SUBJECT PARTICIPATION

All treated patients will be followed for a minimum of 24 months after the ARI-HER2 cell infusion. Following their participation in the ARI-HER2 study, a long-term follow-up study is planned to meet the legal requirements arising from good clinical practices for advanced therapy medicinal products.

5.4 TRACEABILITY

In accordance with good clinical practice guidelines for advanced therapies, a specific procedure will be drawn up to guarantee the traceability of the investigational product.

This document will be distributed to the participating sites and will be included in the investigator's file and master file. The sites will not keep the empty vials/bags of the IMP; destruction will be done following the recommendations of the sponsor, which are the usual ones for gene therapy products that contain genetically modified organisms (GMOs) and whose destruction will be documented according to the protocol of each site and considering the biosafety instructions provided by the sponsor.

5.5 RANDOMIZATION CONTROL AND BLINDING (IF APPLICABLE)

Not applicable. There will be no randomization or blinding in this clinical trial.

5.6 SOURCE DATA IDENTIFICATION

All observations or notes recorded on the clinical interventions, as well as all the reports and notes necessary for the evaluation and reconstruction of the research study will be acknowledged as source documents. Accordingly, source documents include, but are not limited to laboratory reports, ECG traces, X-rays, radiologist reports, hospital reports, participant progress notes, patient diaries, biopsy results, or any other reports or record of any procedure in accordance with this protocol. Whenever possible, the original document should be kept as the source document, however, the contribution of a photocopy is accepted if it is clear, legible and an exact duplicate of the original document.

5.7 BIOLOGICAL SAMPLES

All samples collected from patients during the clinical trial are clearly outlined in the protocol and are essential for the proper evaluation of the treatment's toxicity and efficacy. Samples that do not require real-time processing will be coded and securely stored at each center in a restricted-access location, accessible only to authorized study personnel, until the conclusion of the study. Upon completion of the study, all samples will be destroyed in compliance with the legal requirements specified in Law 14/2017 regarding Biomedical Research. Samples will not be stored in biobanks or human sample collections unless the patient has signed an informed consent form in accordance with RD 1761/2011 (November 18), which regulates the operation and organization of the National Registry of Biobanks for Biomedical Research in Spain ("Registro Nacional de Biobancos para investigación biomédica").

Summary of Samples to be Collected during the Study:

1. Core Laboratory (Hospital Clínic de Barcelona):

The following table (Table 4) outlines the sample collection schedule, including the type, volume, timepoints, and purpose of each sample. Follow instructions for sample collection, numbering, processing, and shipment provided in the sample management guide.

Sample type	Volume/Quantity	Timepoint	Visit*	Use
Mandatory				
Tumor	FFPE or 10 µm	Pre-treatment	First visit (D-42)	Molecular analysis:

biopsy	slides**, 1 HER2 IHC slide, 1 H&E slide. If possible, also fresh frozen tissue.			HER2DX genomic test; histopathological evaluation: HER2 IHC and H&E (sent to the central lab, scanned, and returned)
Blood samples	20 mL of whole blood (2 tubes of 10 mL)	Screening	D-13 to-D7	Peripheral blood cells (PBMCs) for translational research. Plasma for cytokine assessment (4 mL) and ctDNA monitoring (4 mL).
		Treatment period	Days 0, 3, 7, 14, 28 and 84	
		EOT	EOT visit	
	5 mL of whole blood	Treatment period	Days 0, 3, 7, 14, monthly (up to two consecutive assessments with undetectable levels)	ARI-HER2 monitoring (flow cytometry)
	5 mL of whole blood	Treatment period	Days 0, 3, 7, 14, 28, monthly (12M FUP); every 3 months (up to 24M FUP) or until CAR-T is undetectable.	ARI-HER2 monitoring (qPCR)
		EOT	EOT visit	
Optional				
Tumor biopsy	FFPE or 10 μm slides **, 1 HER2 IHC slide, 1 H&E slide. If possible, also fresh frozen tissue.	Treatment period	D28	Molecular analysis: HER2DX genomic test; histopathological evaluation HER2 IHC and H&E (sent to the central lab, scanned, and returned)
		EOT	EOT visit	Molecular analysis, translational research

Table 4. Sample collection plan.

*Specific timepoint flexibility is detailed in Table 8. Visits and procedures calendar.

**Guide for number of 10 µm slides according to the tumor area:

Tumor area ≥100 mm ²	→	1 x 10µm sections
Tumor area = 20-99 mm ²	→	3 x 10µm sections
Tumor area = 7-19 mm ²	→	6 x 10µm sections

Tumor area $\leq 6 \text{ mm}^2$ → $\geq 8 \times 10 \mu\text{m}$ sections

2. Local Laboratory (details in Table 8):

- Peripheral blood samples for:
 - Hematology.
 - Biochemistry.
 - Serology.
 - Tumor markers and inflammation markers.
- Urine samples for:
 - Pregnancy tests.
 - Urine sediment and ionogram.

Future Studies:

Additional biospecimens may be collected for future studies if the patient provides informed consent for participation in the *Collection of Biospecimens of Patients with Solid Tumors at Hospital Clínic* (Dr. Aleix Prat).

Instructions for sample procedures and shipment will be provided to the participating sites in the sample management guide.

6 INCLUSION AND EXCLUSION CRITERIA

6.1 SUBJECT INCLUSION CRITERIA

Subjects are eligible for the study if all the following apply:

1. Able to understand the trial and willingly sign the informed consent.
2. Is ≥ 18 years of age at the time of signing the informed consent.
3. Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1.
4. Histological diagnosis of HER2-positive metastatic or unresectable breast cancer according to American Society of Clinical Oncology guidelines.
5. Locally confirmed HER2[+] status with IHC score 3+ in a newly obtained tumor sample (mandatory pre-treatment biopsy for local confirmation). HER2-positive tumors with IHC 2+ and amplified ISH will not be considered for inclusion.
6. Prior disease progression or intolerance to standard HER2-targeted therapies:
 - a. Patients must have received at least 2 lines of HER2-directed therapy for their metastatic disease, including trastuzumab-deruxtecan (T-DXd). Prior HER2- targeted neo-adjuvant or adjuvant therapy that resulted in relapse within 12 months of the completion of therapy will be considered a line of treatment for metastatic disease.
 - b. Based on the physician's choice, participants' eligibility, and institutional and local guidelines, participants may also have received post-T-DXd therapy, for example, a tucatinib-based regimen and/or T-DM1.
7. At least one measurable and/or evaluable lesion according to RECIST 1.1 criteria.
8. Life expectancy of at least 3 months, in the opinion of the investigator.

9. Adequate organ function determined within 28 days prior to enrollment, defined as follows:

- Hematological (without need for transfusion and or growth factor support within 15 days):
 - Absolute neutrophil count $\geq 1.5 \times 10^9/L$
 - Platelet count $\geq 100,000/mm^3$; participants with stable platelet count from 75,000 to 100,000/mm³ may be included with approval from the medical monitor.
 - Hemoglobin ≥ 9 g/dL
 - Lymphocytes $> 0.1 \times 10^9/L$
- Renal
 - Serum creatinine $\leq 1.5 \times$ ULN or 24-hour creatinine clearance ≥ 50 mL/min (Cockcroft - Gault) for subjects with creatinine levels $> 1.5 \times$ ULN.
- Hepatic
 - Serum bilirubin $\leq 1.5 \times$ ULN OR direct bilirubin $\leq$ ULN for a subject with total bilirubin level $> 1.5 \times$ ULN, except for patients with a diagnosis of Gilbert's syndrome.
 - AST and ALT $\leq 2.5 \times$ ULN OR $\leq 5 \times$ ULN for subjects with liver metastases.
- Coagulation: International normalization ratio (INR) or prothrombin time (PT) $\leq 1.5 \times$ ULN.
- Lung function: CTCAE v5.0 grade ≤ 1 dyspnea and SatO₂ $\geq 92\%$.
- Cardiac: left ventricular ejection fraction (LVEF) $\geq 50\%$ as determined by either echocardiogram or MUGA obtained within 12 weeks before the first dose of study intervention.

10. Last dose of prior anti-cancer therapy at least 28 days or 5 half-lives prior to ARI-HER2 infusion.

11. Participants with history of stable CNS metastases are eligible, provided the following criteria are met:

- Treated brain metastases, in response to local or systemic treatment. If steroid therapy is required for the management of neurologic symptoms, the maximum allowed dose will be prednisone ≤ 10 mg/day or equivalent.
- Radiological stability of CNS metastases on MRI at the time of screening.
- If new brain metastases are diagnosed during screening, the patient may receive local treatment with radiotherapy and undergo an MRI scan 4 weeks after completion. If stability is confirmed, the patient may then proceed to the treatment phase.

12. Adequate venous peripheral access for apheresis.

13. Ability to provide a newly obtained tumor biopsy (mandatory for screening). If subjects have only one or no accessible lesion, their inclusion will be discussed with the research team. Additional biopsies for translational research will be offered to the patients at 1st reevaluation (D28) and progression (EOT).

14. Patients must agree not to participate in another interventional study while receiving study drug (subjects who are currently in the Follow-up Period of an interventional clinical trial are allowed).

6.2 SUBJECT EXCLUSION CRITERIA

Subject will be excluded from participation if any of the following apply:

1. Known sensitivity to any of the products or components to be administered. Contraindication to receive lymphodepleting chemotherapy.
2. Known or suspected leptomeningeal disease or spinal cord compression.
3. Toxic effects of prior therapies not resolved to CTCAE v5.0 grade 1 or less, with the following exceptions: alopecia, neuropathy resolved to $\leq$ Grade 2, fatigue $\leq$ Grade 2, or endocrinopathy $\leq$ Grade 2.
4. Incompatible concurrent medication: systemic therapeutic corticosteroids must be discontinued 72 hours before CAR-T infusion. However, patients who require replacement therapy for adrenal insufficiency may be enrolled if steroid treatment doses do not exceed 10 mg of prednisone or equivalent.
5. History or evidence of symptomatic autoimmune pneumonitis, glomerulonephritis, vasculitis, or other symptomatic autoimmune disease, or active autoimmune disease or syndrome that has required systemic treatment in the past 2 years (e.g., disease-modifying agents, corticosteroids, or immunosuppressive drugs), except for vitiligo or resolved childhood asthma/atopy. Replacement therapy (e.g., thyroxine, insulin, or physiologic corticosteroid replacement therapy for adrenal or pituitary insufficiency) is not considered systemic treatment.
6. Uncontrolled or significant cardiovascular disease including any of the following:
 - a. History of myocardial infarction or unstable angina within 6 months prior to enrollment.
 - b. History of symptomatic congestive heart failure (New York Heart Association Class II to IV)
 - c. Ventricular arrhythmia requiring therapy
7. Previous history of allogeneic hematopoietic cell or solid organ transplantation.
8. Active infection within 14 days prior to study treatment.
9. Known history of human immunodeficiency virus (HIV) infection.
10. History of active tuberculosis.
11. Positive Hepatitis B virus antibodies and surface antigen (indicating acute HBV or chronic HBV) or Hepatitis C (HCV RNA (qualitative)). Hepatitis C RNA testing is not required in subjects with negative Hepatitis C antibody testing. Hepatitis B virus antibodies are not required in subjects with negative Hepatitis B surface antigen.
12. History of other malignancy within the past 2 years with the following exceptions at the time of enrollment: adequately treated non-melanoma skin cancer without evidence of disease, adequately treated cervical carcinoma in situ without evidence of disease, adequately treated breast ductal carcinoma in situ without evidence of disease, prostatic intraepithelial neoplasia without evidence of prostate cancer or adequately treated superficial or in-situ carcinoma of the bladder without evidence of disease.
13. Live vaccine against infectious diseases administered within 28 days of study treatment.
14. Unlikely to comply with all protocol-required study visits or procedures, to the best of the subject and investigator's knowledge.
15. History or current evidence of any condition, therapy, or laboratory abnormality that might confound the results of the trial, interfere with the subject's participation for the full duration of the trial, or is not in the best interest of the subject to participate, in the opinion of the treating investigator.
16. If WOCBP or men, refusal to the use of approved contraceptive methods (e.g. barrier

device, intrauterine device, abstinence) during the study and for at least 12 months following the last dose of the study cell infusion and until no CAR-T cells can be detected after two consecutive PCR tests.

17. Pregnant or breast-feeding women.

6.3 TREATMENT SUBJECTS

The treatment is targeted for adult patients with refractory or relapsed metastatic HER2-positive breast cancer. It is anticipated that 9 to 12 patients will be infused with ARI-HER2. The study will be conducted in 3 patient cohorts, each consisting of 3 patients. However, to accommodate potential dose-limiting toxicity (DLT), 3 additional patients may be included if necessary, ensuring proper evaluation of the treatment's safety.

6.4 PROCEDURES FOR SUBJECT'S SELECTION

Patients who meet selection criteria will be offered to participate in the study. Each patient will be informed about the objectives, risks and benefits derived from its participation in the study; patients will also be informed about their right to withdraw from the study at any point without any prejudice. Following the discussion with the specialist, patients will be asked to sign an informed consent form to confirm their participation. No additional promotional materials (advertisements or publicity) will be used to recruit participants.

6.5 PATIENT ENROLLMENT PROCEDURES

Participants must be registered in the eCRF after signing the ICF, enrollment confirmation will be provided by the Sponsor Medical Monitor after reviewing inclusion and exclusion criteria, both before performing apheresis and before initiating lymphodepleting chemotherapy. Issues causing treatment delays should be discussed with the Local Principal Investigator and Sponsor/SOLTI. If a participant does not receive protocol therapy following registration within the allowed period, the participant's registration in the study may be cancelled. Sponsor and SOLTI should be notified cancellations as soon as possible.

The registration procedures are as follows:

- 1) All sites should write an e-mail to CRA and SOLTI HQ staff to notify them of the upcoming registration and slot availability request with CC to Sponsor Medical Monitor.
- 2) Obtain written informed consent for the study. Investigator assessment of the patient's potential eligibility for the study should be evaluated beforehand.
- 3) After signing the ICF, tumor tissue newly obtained will be analyzed at local laboratory for HER2 status determination and then sent to central lab for molecular analysis.
- 4) Complete the protocol-specific eligibility checklist using the eligibility assessment documented in the participant's medical/research record. To be eligible for registration to the study, the participant must meet all eligibility criteria listed in the checklist.
- 5) After all inclusion and exclusion criteria are reviewed by the investigator or qualified designee to ensure that the participant qualifies for the trial, an inform will be sent together with a completed study-specific Patient Enrollment Form to Sponsor Medical Monitor for evaluation with CC to CRA and SOLTI Staff.
- 6) E-mail the following anonymized documents and screening sample results, and the Patient Enrollment Form to the Medical Monitor with CC to CRA and SOLTI Staff.
Copies of laboratory, imaging, and pathology reports. Summary physician notes.
- 7) The decision will be available within 24 hours, confirming whether the patient might be allocated. The investigator will maintain a screening log to record details of all patients screened and to confirm eligibility or record reasons for screening failure, as applicable.
- 8) Once registration confirmation from Sponsor medical monitor is received, proceed with treatment.

7 STUDY TREATMENT

7.1 SUMMARY OF TRIAL TREATMENTS

The pharmacological treatments to be used in this trial are outlined below in Table 5.

Table 5. Trial Pharmacological Treatment administration summary

Treatment	Dosage regimen	Route	Type IMP/AUX	Regulatory situation
INVESTIGATIONAL MEDICINAL PRODUCT				
ARI-HER2 cells	Cohort 1: 1.5 x 10 ⁶ cell/kg (3 fractions): 0.15x10 ⁶ cell/Kg (10%), 0.45x10 ⁶ cell/Kg (30%), 0.90x10 ⁶ cell/Kg (60%)	iv	IMP	Under investigation
	Cohort 2: 3 x 10 ⁶ cell/kg (3 fractions): 0.30x10 ⁶ cell/Kg (10%), 0.90x10 ⁶ cell/Kg (30%), 1.80x10 ⁶ cell/Kg (60%)			
	Cohort 3: 4.5 x 10 ⁶ cell/kg (3 fractions): 0.45x10 ⁶ cell/Kg (10%), 1.35x10 ⁶ cell/Kg (30%), 2.70x10 ⁶ cell/Kg (60%)			
AUXILIARY MEDICATION				
Previous medication to IMP (QTLD)				
Fludarabine	30 mg/m ² /d d-6, d-5, d-4 before infusion	iv	AUX	authorized
Cyclophosphamide	300 mg/m ² /d d-6, d-5, d-4 before infusion	iv	AUX	authorized
<i>Bendamustine*</i>	100 mg/m ² /day on days -5 and -4	iv	AUX	authorized
Ondansetron	4-8 mg each 8 hours	iv	AUX	authorized
Medication during IMP-infusion				
Paracetamol	15 mg/kg (max 1 g/dose)	iv or po	AUX	authorized
Antihistaminic	i.e Polaramine 5mg	iv or po	AUX	authorized
Medications post-infusion (recommended anti-infectious prophylaxis)**				
Levofloxacin	500 mg each 24 hours	po	AUX	authorized
Fluconazole	400 mg each 24 hours	po	AUX	authorized
Acyclovir	400 mg each 12 hours	po	AUX	authorized
Cotrimoxazole***	800 mg each 24 hours	po	AUX	authorized
G-CSF (Filgrastim)	300-480 ug each 24 hours	IV	AUX	authorized
Medications post-infusion (in case of AEs)				
Tocilizumab (if CRS)	8 mg/kg if >30 kg (maximum 800 mg dose)	iv	AUX	authorized
Dexamethasone (if ICANS)	0.2 mg/kg/day q8-12h	iv or po	AUX	authorized
Allopurinol****	300 mg/24h	po	AUX	authorized

* only in case of contraindication to cyclophosphamide and fludarabine.

**this treatment may be individualized for each patient according to investigator criteria.

***pentamidine can be used as an alternative to cotrimoxazole.

****medical criteria

7.2 ARI-HER2: INVESTIGATIONAL MEDICINAL PRODUCT ADMINISTRATION

The treatment to be administered is ARI-HER2 cells: adult autologous differentiated T-cells extracted from peripheral blood, expanded and transduced with a lentivirus containing a low-affinity chimeric antigen receptor anti-HER2 (4D5.5) conjugated with 4-1BB and CD3z. ARI-HER2 will be administered intravenously under a split regime. A total dose between 0.15 and 4.5 x10⁶ cell/Kg will be administered in 3 administrations (fractions). Ideally these 3 fractions will be administered within a period of 12 days.

Total dose can be 1.5, 3 or 4.5 x10⁶ cell/Kg depending on cohort assigned, as detailed below (Table 6):

Cohort	Total dose	Fraction 1: 10% (aliquot)	Fraction 2: 30% (aliquot)	Fraction 3: 60% (aliquot)
1	1.5x10 ⁶	0.15x10 ⁶ (0.15)	0.45x10 ⁶ (0.45)	0.9x10 ⁶ (0.9)
2	3x10 ⁶	0.3x10 ⁶ (0.15+0.15)	0.9x10 ⁶ (0.9)	1.8x10 ⁶ (1.8)
3	4.5x10 ⁶	0.45x10 ⁶ (0.45)	1.35x10 ⁶ (0.9+0.45)	2.7x10 ⁶ (0.9+1.8)

Table 6. Dose escalation for ARI-HER2

For additional details regarding IMP see investigational brochure (ARI-HER2 IB) and section 2.3 of the current protocol.

Once acceptance criteria are met, and the patient has completed lymphodepleting chemotherapy (see section 7.3, auxiliary medication), the first fraction of ARI-HER2 cell will be delivered to the patient's room and thawed *in situ* in a water bath heated at 36-38°C. The same procedure will be repeated for second and third fraction provided there are not signs or symptoms of CRS/ICANS requiring specific medical intervention.

Patient will receive ARI-HER2 in three different days (1 fraction per day; maximum 3 fractions). Before each administration the following steps must be followed:

1. **Premedication**, 30 minutes before the ARI-HER2 infusion, consisting in paracetamol 1g + dexchlorpheniramine 5 mg.
2. **ARI-HER2** will be administered at a rate of 10-20 ml/min. The volume of infusion is 30 ml, so the infusion usually takes 15-30 minutes. Afterwards, the system must be flushed with saline to ensure that the entire cell dose is administered. For obvious reasons, leukodepletion filters cannot be used.
3. **In case of infusion reaction**: premedication can be repeated and a NSAID can be added (i.e. dexketoprofen, naproxen), but systemic corticosteroids (i.e. prednisone,

methylprednisolone or dexamethasone) should be avoided if allowed by the clinical situation. If a corticosteroid must be administered, then hydrocortisone is recommended. A crash cart must be available in the ward in case of severe allergic reaction or severe hypotension.

Vital signs will be monitored before and after the infusion (temperature, respiratory rate, pulse, and blood pressure). Patients will remain hospitalized for a minimum of 7 days starting from the last infusion of ARI-HER2.

7.3 AUXILIARY AND CONCOMITANT MEDICATION

7.3.1 Before infusion

Lymphodepleting chemotherapy is required before ARI-HER2 infusion and will be composed of fludarabine 30 mg/m²/day (90 mg/m² total dose) and cyclophosphamide 300 mg/m²/day (900 mg/m² total dose), both on days -6, -5 and -4. In case of contraindication to any of these agents, bendamustine 100 mg/m²/day on days -5 and -4 will be used instead. Of note, patients in whom ARI-HER2 cell infusion is delayed beyond 4 weeks for medical reasons will require a second round of lymphodepleting chemotherapy.

7.3.2 Infusion days

Ondansetron (4-8 mg IV every 8 hours × 3 days) will be given for nausea, or as per standard of care at the treating institution.

30 minutes before each fraction of ARI-HER2, patients will receive paracetamol 1 g and antihistamine (i.e. Polaramine 5 mg). In the rare event of infusion-related reactions, the same medication may be repeated, or a Nonsteroidal Anti-Inflammatory Drug (NSAID) added (e.g. naproxen). If a corticosteroid is necessary, hydrocortisone would be the ideal choice.

Other drugs may be given IV per institutional standards if severe chills are developed by the patient.

Filgrastim 5 µg/kg/day administered by subcutaneous injection, or as per standard of care at the treating institution. This will be administered each day from day +2 or when absolute neutrophil count < 500/mm³, until the absolute neutrophil count reaches >500-1000/mm³.

7.3.3 Prophylactic medication

All patients may receive the following schedule of prophylactic medication:

- Levofloxacin 500 mg each 24 hours (orally), from day +1 from infusion or when absolute neutrophil count < 500/mm³, until peripheral neutrophil count >500-1000 cells/mm³. Escalating antibiotherapy should be considered if required by the clinical situation.
- Fluconazole 400 mg each 24 hours (orally), from day +1 from infusion or when absolute neutrophil count < 500/mm³, until peripheral neutrophil count >500-1000 cells/mm³. Escalating prophylaxis to isavuconazole or posaconazole must be considered for patients treated with DXM at high doses (e.g. for management of

CRS/ICANS) (see section 7.4).

- Acyclovir 400 mg each 12 hours (orally), from day +1 or when absolute lymphocyte count < 500/mm³, at least for 6-12 months and/or CD4 counts >200 cells/mm³.
- Cotrimoxazole 800 mg daily (three times per week, orally), from day +1 or when absolute lymphocyte count < 500/mm³, at least for 6-12 months and/or CD4 counts >200 cells/mm³.
- Allopurinol 300 mg each 24 hours (orally), from day +1 until day +30 from infusion.

7.3.4 Permitted concomitant medications

All treatments that the investigator considers necessary for a participant's welfare may be administered at the discretion of the investigator keeping with the community standards of medical care. All concomitant medication will be recorded on the case report form (CRF) including all prescriptions, over-the-counter (OTC), herbal supplements, and IV medications and fluids. If changes occur during the trial period, documentation of drug dosage, frequency, route, and date may also be included on the CRF.

All concomitant medications received within 28 days before the first dose of trial treatment and 28 days after the last dose of trial treatment should be recorded. Concomitant medications administered after 28 days after the last dose of trial treatment should be recorded for SAEs reporting. The following concomitant treatments are permitted during the study:

- Erythropoiesis-stimulating agents (ESA) are allowed (such as Procrit®, Aranesp®, and Epogen®) for the supportive treatment of anemia. Blood transfusions are permitted during the study.
- The use of medication for the treatment of diarrhea, nausea, or vomiting is permitted.
- Any medications deemed necessary to ensure patient safety and well-being may be administered at the discretion of the investigator except for prohibited therapies contained in the next section.
- Single dose palliative radiotherapy for control of pain, cough or bleeding is permitted if it is applied to a non-target lesion or a target lesion if it is not the only target lesion for RECIST evaluation.

7.3.5 Prohibited concomitant medications

Medications or vaccinations specifically prohibited in the exclusion criteria are not allowed during the ongoing trial (see section 5.2). If there is a clinical indication for one of these or other medications or vaccinations specifically prohibited during the trial, discontinuation from trial therapy may be required. Moreover, emphasis may be made on communicating this information to Medical Monitor and the coordinating investigator before any of those therapies are initiated. In controversial situations about the use of theoretically prohibited therapy, it should be communicated and discussed with Medical Monitor and coordinating investigator before they are administered to avoid patient discontinuation.

Patients are prohibited from receiving the following medications during the treatment phase of this study and/or after receiving ARI-HER2 cells infusion:

- Other anticancer therapies, such as extensive radiotherapy, hormone therapy or any other HER2-targeted therapy, and including alternative or complementary therapies that could be active against cancer, such as herbal medicines.
- Any other investigational therapy.
- Systemic steroid therapy (equivalent to 10 mg of prednisone per day) and other immunosuppressive drugs are prohibited, except for the management of AEs related to the study treatment (CRS, ICANS), according to the instructions given in Section 2.3. All treatments that the Investigator considers necessary for a participant's welfare may be administered at the discretion of the Investigator in keeping with the community standards of medical care.
- Vaccines will not be allowed during the clinical trial, except for inactive vaccines during the follow-up period considering the benefit/risk balance for each patient.
- Immunosuppressive drugs such as (but not restricted to): cyclosporine, tacrolimus, methotrexate, mycophenolate mofetil, sirolimus, alemtuzumab, or daclizumab are especially prohibited.
- In addition, T-Lymphocytes are especially susceptible to chemotherapeutics commonly used with hematologic malignancies, such as (but not restricted to): cyclophosphamide, fludarabine, bendamustine, anthracyclines, etoposide or vincristine so. These treatments are forbidden after the administration of ARI-HER2 cells. Very occasionally, some of these agents (e.g. cyclophosphamide) may be used for the treatment of severe CRS that is refractory to all conventional measures.

7.3.6 Treatment delays

No dose reductions are allowed in any of the drugs involved in the treatment, only dosing delay will be permitted.

In case of unacceptable toxicity (grade ≥ 3 adverse effects related to HER2 CAR-T infusion) after administration of any of the fractions, the remaining fractions will not be administered.

Administration variability of 1-7 days after each dose administered would be acceptable for clinical or logistical reasons. Ideally, all 3 dose fractions would be administered within 12 days.

7.4 GENERAL AES MANAGEMENT

The administration of the second and/or third fraction will be avoided in case of any of the following situations after the previous dose (grades are defined according to CTCAE 5.0 criteria).

- Grade ≥ 3 CRS or ≥ 2 ICANS (see Table 7). In the case of grade 2 CRS or grade 1 ICANS, the administration of further ARI-HER2 fractions should be discussed with the medical monitor.

- Clinical and/or microbiological evidence of an active infection*.

*It should be noted that cytopenias, often occurring alongside fever and/or elevated blood inflammatory markers, are expected after lymphodepleting chemotherapy and do not necessarily indicate an active infection. These findings do not require discontinuation of treatment and will be thoroughly discussed by the research team and/or medical monitor to individualize decisions based on each patient's clinical situation.

- Potentially severe on-target off-tumor toxicity, defined as: grade ≥ 3 diarrhea, grade ≥ 3 elevation of liver enzymes (ALT, AST), grade ≥ 2 dyspnea or hypoxia, especially in the presence of radiological signs of pneumonitis and/or echocardiographic confirmation of

ejection fraction decrease, and/or any other grade ≥ 3 toxicity at investigator criteria. If tumor lysis syndrome is suspected, hydration and other conventional measures (e.g. allopurinol 300 mg/24 h for a duration ranging from 7 days to one month depending on the presence/absence of cell expansion) may be used at medical discretion.

Table 7 establishes the recommended management of potential AEs according to the toxicity grade:

Table 7. AEs management

Toxicity grade	Medication and global management	ARI-HER2 management
Cytokine release syndrome (CRS)		
Grade 1 (fever [≥37.8°C] without hypotension or hypoxia)	Consider tocilizumab 8mg/kg (max 800 mg) if persistent fever (>24h). Tocilizumab can be repeated after 24h. If no response after 2 doses, consider dexamethasone (DXM) IV 10mg/6h. Supportive therapy (fluids, antipyretics). Close monitoring in an intermediate care ward.	Continue with fractions of ARI-HER2 after 36-48h without fever.
Grade 2 (fever + hypotension not requiring vasopressors and/or hypoxia responding to low-flow oxygen)	Tocilizumab 8mg/kg (max 800 mg) + DXM IV 10mg (repeat both drugs if no improvement after 8 h) + supportive therapy if not initiated. If no response after first 2 doses, continue DXM IV 10mg/6h. Alert local intensive care unit (ICU).	Continuing ARI-HER2 should be discussed with the medical monitor (MM).
Grade 3 (fever + hypotension requiring 1 pressor and/or hypoxia requiring high-flow oxygen)	Manage in ICU. If no response to 2 doses of tocilizumab 8mg/kg + DXM IV 10mg/6h, consider anakinra 8mg/kg each 24h. If no response to anakinra, consider single dose of siltuximab IV 11 mg/kg. Individualize further intensive care.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 4 (fever + hypotension requiring >1 pressor and/or hypoxia requiring ventilation/intubation/positive pressure)		
Immune-effector cell-associated neurotoxicity syndrome (ICANS)		
Grade 1 (ICE* 7-9, mild encephalopathy, awakens spontaneously; no seizures, no motor findings, no cerebral edema).	Start DXM IV 10mg/6h. Perform EEG, lumbar puncture and neuroimaging (MRI); rule out CNS infection. Monitor closely (ICE every 6-8 hours). Individualize supportive care (oral intake, raised head at 30°).	Continuing ARI-HER2 must be discussed with MM.
Grade 2 (ICE* 3-6, awakens to voice; no seizures, no motor findings, no cerebral edema).	Transfer to ICU. If no improvement after 24-48 h with DXM IV 10mg/6h, escalate to DXM IV 20mg/6h. If no response after 24-48 h, start anakinra (8mg/kg each 24h). If no response after 24h, consider single dose of siltuximab IV 11 mg/kg. Individualize further neurointensive treatment. In refractory cases, consider dasatinib 140mg/24h to neutralize CAR activity and/or other individualized measures (plasmapheresis, intrathecal treatment).	Avoid further fractions of ARI-HER2. Alert MM.
Grade 3 (ICE* 0-2, awakens to tactile stimulus; any clinical seizure or epileptic activity on EEG that resolves with intervention).		
Grade 4 (stupor/coma, unable to perform ICE*; prolonged [≥5 min] or repetitive clinical or electrical seizures; focal motor weakness; diffuse cerebral edema or papilledema, decerebrate/decorticate posturing, cranial nerve VI palsy, Cushing triad).		
Immune-effector cell-associated hemophagocytic-like syndrome (IEC-HS)		

Grade 2 (not severe clinical manifestations).	Anakinra 8mg/kg each 24h. Start DXM IV 10mg/6h if not initiated. Consider transfer to ICU. If no response, consider single dose of siltuximab IV 11 mg/kg. In refractory cases, consider alternative agents (ruxolitinib, low-dose etoposide).	Avoid further fractions of ARI-HER2. Alert MM.
Grade 3 (severe, coagulopathy with bleeding requiring transfusion, acute kidney injury, hypotension, respiratory distress).		
Grade 4 (intubation, dialysis, intensive intervention required, life-threatening consequences).		
ON-TARGET OFF-TUMOR TOXICITY (AEs related to damage of HER2-expressing tissues)		
Cardiotoxicity (heart failure, LVEF decreased, elevated cardiac markers)		
Grade 1 (increased BNP/pro-BNP without symptoms of heart failure; asymptomatic increase of troponin I)	Close clinical monitoring. Perform echocardiogram and ECG. Repeat biomarkers within 48-72 hours. Consider cardiology consultation.	Consult with MM. Consider interrupting ARI-HER2 if no improvement.
Grade 2 (symptoms of heart failure with moderate activity; resting LVEF 40-50% or 10-19% drop from baseline)	Consider hospital admission and cardiology consultation. Consider diuretic drugs and cardioprotective therapy.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 3 (symptoms of heart failure at rest or with minimal activity; resting LVEF 20-39% or ≥20% drop from baseline; increased troponin I levels consistent with myocardial infarction)	Hospital admission, consider transfer to ICU. Specific supportive therapy as required. Consider steroids and/or other immunosuppressive drugs. In refractory cases, consider dasatinib 140mg/24h and/or high-dose cyclophosphamide.	
Grade 4 (life-threatening heart failure / myocardial infarction requiring urgent intervention or hemodynamic support; resting LVEF <20%)		
Pulmonary toxicity (hypoxia, dyspnea, pneumonitis/interstitial lung disease)		
Grade 1 (incidental findings on CT scan, without hypoxia, dyspnea or any other respiratory symptoms)	Close monitoring, perform high-resolution chest CT scan, consider spirometry. Consider low-dose steroid therapy.	Continuing ARI-HER2 must be discussed with MM.
Grade 2 (dyspnea or hypoxia [SO ₂ <90%] with moderate activity, limiting instrumental ADL; requirement of intermittent supplemental oxygen)	Hospital admission, consider transfer to ICU for grade ≥3. Consult pneumology specialist. Consider steroids and/or other immunosuppressive drugs. In refractory cases, consider dasatinib 140mg/24h and/or high-dose cyclophosphamide.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 3 (dyspnea or hypoxia [SO ₂ <90%] with minimal activity / at rest, limiting self-care ADL; oxygen required)		
Grade 4 (life-threatening respiratory compromise, intubation or any other urgent intervention indicated)		
Hepatotoxicity (transaminase and/or bilirubin elevation following ARI-HER2 infusion)		
Grade 1 (ALT/AST > ULN to 3x ULN or baseline [if abnormal]; blood bilirubin > ULN to 1.5x ULN or baseline)	Close monitoring (every 24-48 hours). Repeat viral serologies, consider imaging and differential diagnosis with other causes of acute hepatitis in	Continue ARI-HER2.

Grade 2 (ALT/AST > 3-5x ULN or baseline; blood bilirubin > 1.5-3x ULN or baseline)	immunocompromised patients. Consider hepatologist consultation.	Continuing ARI-HER2 must be discussed with MM.
Grade 3 (ALT/AST > 5-20x ULN or baseline; blood bilirubin > 3-10x ULN or baseline)	Consider hospital admission and early specialist consultation. Initiate steroids and/or other immunosuppressive drugs. In refractory cases, consider dasatinib 140mg/24h and/or high-dose cyclophosphamide.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 4 (ALT/AST > 20x ULN or baseline; blood bilirubin > 10x ULN or baseline)		
Gastrointestinal toxicity (diarrhea, colitis/enterocolitis, vomiting)		
Grade 1 (increase of <4 stools/day over baseline, mild nausea/vomiting)	Close monitoring and symptomatic management.	Continue ARI-HER2.
Grade 2 (increase of 4-6 stools/day over baseline, limiting instrumental ADL; vomiting requiring outpatient hydration)	Stool testing for enteropathogens, <i>C. difficile</i> toxin and calprotectin. Consider endoscopic studies and hospital admission.	Continuing ARI-HER2 must be discussed with MM.
Grade 3 (increase of ≥7 stools/day over baseline, limiting self-care ADL; vomiting requiring intravenous hydration or limiting oral intake; mucus or blood in stools; severe or persistent abdominal pain; peritoneal signs)	Hospital admission, consider transfer to ICU. Early gastroenterologist consultation. Consider steroids and/or other immunosuppressive drugs. In refractory cases, consider dasatinib 140mg/24h and/or high-dose cyclophosphamide.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 4 (life-threatening consequences, urgent intervention indicated)		
Pancreatitis		
Grade 2 (asymptomatic elevation of pancreatic enzymes)	Close monitoring. Consider abdominal CT scan. Repeat amylase and lipase determination in 48-72 hours.	Continuing ARI-HER2 must be discussed with MM.
Grade 3 (abdominal pain, vomiting; analgesia, nutritional support or any other medical intervention indicated)	Hospital admission, consider transfer to ICU. Early gastroenterologist consultation. Consider steroids and/or other immunosuppressive drugs. In refractory cases, consider dasatinib 140mg/24h and/or high-dose cyclophosphamide.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 4 (life-threatening consequences, urgent intervention required)		
Bone toxicity (accelerated osteopenia/osteoporosis, hypercalcemia, fragility fractures)		
Grade 1 (bone mineral density [BMD] t-score -1 to -2.5; asymptomatic fracture as radiological finding; corrected serum Ca > ULN-11.5 mg/dl)	If osteopenia, initiate vitamin D and calcium supplementation (if not hypercalcemic). Encourage exercise and monitor BMD. Treat hypercalcemia if present (oral hydration, review and discontinue medications that increase calcium), monitor every 48-72 hours.	Continue ARI-HER2.
Grade 2 (BMD t-score < -2.5, loss of height <2 cm; symptomatic [not displaced] fracture, immobilization indicated; corrected serum Ca 11.5-12.5 or symptomatic hypercalcemia)	Treat hypercalcemia if present (IV hydration, consider antiresorptive drugs); consider admission and calcium testing every 24 hours.	Continuing ARI-HER2 must be discussed with MM.

Grade 3 (corrected serum Ca 12.5-13.5 mg/dl, hospitalization indicated; severe/displaced bone fracture limiting self-care ADL)	Hospital admission and early specialist consultation; consider transfer to ICU. Treat hypercalcemia if present (aggressive IV hydration, bisphosphonate IV, calcitonin and/or denosumab, dialysis in severe cases). In refractory cases, consider immunosuppressive drugs, dasatinib 140mg/24h and/or high-dose cyclophosphamide.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 4 (corrected serum Ca > 13.5 mg/dl, life-threatening consequences; urgent intervention indicated)		
Other toxicities considered to be related to damage of HER2-expressing tissues		
Grade 1 (asymptomatic or mild symptoms, intervention not indicated)	Close monitoring, symptomatic treatment and/or specific supportive measures if required. Consider complementary procedures for differential diagnosis. Consider hospital admission.	Consult MM to align on individualized management.
Grade 2 (moderate symptoms, not limiting self-care ADL; local or non-invasive intervention indicated)		
Grade 3 (severe and/or significant clinical manifestations, limiting self-care ADL; admission or prolongation of existing hospitalization indicated)	Hospital admission, consider transfer to ICU. Early specialist consultation. Consider steroids and/or other immunosuppressive drugs. In refractory cases, consider dasatinib 140mg/24h and/or high-dose cyclophosphamide.	Avoid further fractions of ARI-HER2. Alert MM.
Grade 4 (life-threatening consequences, urgent intervention indicated)		
AEs related to lymphodepleting chemotherapy		
<i>Neutropenia</i>		
Grade 1 (>1500/mm ³).	Not indicated.	Do NOT suspend ARI-HER2 fractions
Grade 2 (1000-1500/mm ³).		
Grade 3 (500-1000/mm ³).		
Grade 4 (<500/mm ³).	Initiate G-CSF (Filgrastim 300-480 ug/d) + prophylactic levofloxacin 500mg/d and fluconazole 400mg/d until neutrophils >500-1000/mm ³ . Consider escalating antibiotics if fever. Intensify anti-fungal prophylaxis to posaconazole or isavuconazole for patients receiving high doses of DXM and/or other immunosuppressants.	
<i>Anemia</i>		
Grade 1 (>10g/dL).	Not indicated.	Do NOT suspend ARI-HER2 fractions
Grade 2 (8-10g/dL).		
Grade 3 (<8g/dL).	Blood transfusion if Hb <7g/dL. Individualize for Hb 7-8 g/dL. Irradiated products should be used for transfusion. Consider erythropoietin in refractory/persistent cases.	
Grade 4 (life-threatening).	Blood transfusion and support therapy. Irradiated products should be used for transfusion.	Discuss with MM
<i>Thrombopenia</i>		
Grade 1 (>75000/mm ³).	Not indicated. Consider suspending prophylactic anticoagulation if initiated.	Do NOT suspend ARI-HER2 fractions
Grade 2 (50000-75000/mm ³).		
Grade 3 (25000-50000/mm ³).		

Grade 4 (<25000/mm ³).	Consider platelet transfusion if <20000/mm ³ or active bleeding. Irradiated products should be used for transfusion. Consider eltrombopag 100-150mg/24h in refractory/persistent cases.	
<i>Lymphopenia</i>		
Grade 1 (>800/mm ³).	Not indicated.	Do <u>NOT</u> suspend ARI-HER2 fractions
Grade 2 (500-800/mm ³).		
Grade 3 (200-500/mm ³).	Initiate prophylaxis with acyclovir 400mg/12h vo + cotrimoxazole 800mg/24h at least for 6-12 months and until CD4 count >200/mm ³ .	
Grade 4 (<200/mm ³).		

***ICE score for neurological toxicity assessment**

Test	Points
Orientation: year, month, city, hospital	4
Naming: ability to name three objects	3
Following commands: ability to follow simple commands	1
Writing: ability to write a standard sentence	1
Attention: ability to count backwards from 100 to 0 by 10	1

7.5 EARLY DETECTION OF ON-TARGET OFF-TUMOR TOXICITIES

Given the potential risks associated with HER2 targeting, systematic procedures will be implemented to enable prompt identification and management of early toxicities. These procedures complement the scheduled study assessments and are designed to ensure consistent application across sites, standardized reporting, and timely intervention.

1. Cardiotoxicity (heart failure, decrease in LVEF)

- Biomarkers: high-sensitivity troponin and NT-proBNP will be assessed at screening, D0, D7, D14, D28, and then monthly until EOT.
- Imaging: echocardiogram –already required at baseline– will be performed at D28, month 3, month 6, and then every 6 months until EOT, or whenever cardiac symptoms occur.
- Trigger criteria: alterations in ECG, cardiac biomarkers (troponin above ULN) or LVEF <50% (or >10% drop from baseline) will trigger urgent cardio-oncology consultation and specific management as per protocol.

2. Pulmonary toxicity:

- Pulse oximetry will be assessed at every visit.
- Spirometry and high-resolution chest CT scan will be performed if new respiratory symptoms (cough, dyspnea) or hypoxemia occur after treatment, or if imaging abnormalities are found during routine radiologic assessments.
- Oxygen saturation <92%, new-onset dyspnea or suspicious infiltrates will trigger urgent pneumology consultation and specific management as per protocol.

3. Digestive toxicity:

- Periodic clinical monitoring and laboratory tests will allow the prompt detection and specific management of treatment-related diarrhea, other gastrointestinal AEs, as well as hepatic or pancreatic toxicity.

4. Other toxicities:

- Renal function will be assessed at each scheduled blood test.
- Serial bone densitometry assessment (at least at baseline and at month 3) will be performed to detect potential loss in bone mineral density.
- Systematic clinical anamnesis and examination at each visit will allow the detection of other potential AEs related to off-tumor HER2 targeting.

To ensure uniformity across institutions, all site staff will receive training on the early detection of toxicities, and eCRF will include specific fields for cardiac markers, echocardiograms, pulse oximetry and pulmonary assessments.

7.6 COMPLIANCE TO TREATMENT

IMP is administered intravenously in hospitalized patients, so no additional measures to ensure compliance are needed.

7.7 DRUG ACCOUNTABILITY

Each cellular product belongs to a specific patient; only autologous use of ARI-HER2 is permitted. After its administration the containing bag will be immediately destroyed so no accountability will be done by monitor. Traceability must be guaranteed. Sponsor will provide a specific procedure that permits to check the maintenance of traceability.

7.8 MONITORING

A team of monitors designated by the sponsor will monitor the study periodically to ensure that the rights and well-being of the patients are safeguarded, that the protocol, applicable regulations, and ethical requirements are complied with, that all the necessary documentation is available, and that the data collected accurately reflects the data in the CRF. The investigator should allow the monitor to have access to the subjects' medical records, study records, case report forms (CRF), and original signed Informed Consent forms, as well as all original study data documents. Any deficiencies observed during the monitoring visits will be discussed with the investigator and agreement will be reached on what corrective actions should be taken.

7.9 DISCONTINUATION CRITERIA

The end date of the study is expected to coincide with the last visit of the last treated patient; however, as this is a first-in-human study and therefore the level of uncertainty is high, early termination conditions have been established.

7.9.1 Study premature discontinuation

Adverse events are expected in this study in view of the target population (R/R HER2+ metastatic breast cancer), and some of them may be severe (SAEs).

No intermediate analyses are planned for futility evaluation, although criteria for premature termination of the clinical trial due to safety concerns have been defined.

Pre-specified criteria for premature study discontinuation would be any of the following:

- (i) Exitus or permanent organ injury related to the procedure in 2 of 3 patients from the same cohort during the first 28 days (DLT criteria).
- (ii) Uncontrolled T cell neoplasia secondary to CAR-T therapy in one or more patients.
- (iii) The Coordinator, Sponsor or Funding Agencies decides not to pursue the development of ARI-HER2.
- (iv) Decided by the sponsor in the following cases:

- i. If new information on the product leads to doubt as to the benefit/risk ratio.
 - ii. Poor adherence to protocol and regulatory requirements.
 - iii. If the total number of patients are included earlier than expected.
 - iv. Insufficient number of subjects included in the study.
- (v) Other reasons for study discontinuation would be identification of problems in the cell product, systematic protocol violations or inadequate data collection.

7.9.2 Premature end of follow up for individual subjects

7.9.2.1 Patient withdrawal

The withdrawal of the subjects, as well as their reasons, will be reflected both in the clinical history and in the data collection notebook prospectively. Participants have the right to withdraw consent at any time during the study without prejudice. Those patients who decide to withdraw from the study will be asked for permission to establish follow-up by telephone contact. All patients who withdraw/early discontinue from the study will be asked to complete the Early Termination Visit (ETV).

7.9.2.2 Premature discontinuation of patient follow-up

The following reasons have been prespecified as causative factors for premature discontinuation of patient follow-up:

- a) He/she develops any medical condition preventing him/her from receiving ARI- HER2 cells (e.g. concurrent condition requiring corticoids or immune suppressors);
- b) The protocol cannot be followed due to lost to follow-up, investigator's decision (e.g. irreversible clinical deterioration), patient's incomppliance with therapy or medical visits, patient's pregnancy, patient's decision, rapid disease progression requiring alternative therapy, or grade 3-4 toxicity requiring study discontinuation. Patient discontinuation and its reason will be documented in the medical notes and the CRF. Only patients not receiving ARI-HER2 cells may be replaced for the purpose of the study.

All patients who early discontinue from the study will be asked to complete the Early Termination Visit (ETV).

7.9.2.3 Participant's replacement

Only those subjects who have not received any dose of ARI-HER2 can be replaced, as well as those who, having signed consent, prove to be selection failures.

Subjects withdrawing from the study after dosing with ARI-HER2 will not be replaced.

All patients included in the study who received the dose of ARI-HER2, whether they completed the entire study or not, will be included in the analysis. Meanwhile, if the patient does not withdraw consent for data collection, data will be collected from:

- a) Graft of ARI-HER2 cells, until they are no longer detectable
- b) Progression-free survival, until the time of progression or death
- c) Overall survival, until the moment of death

7.9.2.4 Stopping Rules and Safety Oversight

At the occurrence of any serious or unexpected toxicity, the investigator must notify the medical monitor within 24 hours. The MM will review the case and, if appropriate, convene an ad-hoc DSMB meeting outside the scheduled reviews. A written DSMB recommendation (continue, modify, suspend, or terminate the trial) will be communicated to all sites before enrolment is resumed.

Any of the following situations occurring in the first cohort will be considered pre-specified criteria for premature study discontinuation:

1. Any treatment-related death (grade 5 SAE judged related to ARI-HER2).
2. Any life-threatening (grade 4) CRS, ICANS or IEC-HS that does not resolve to grade ≤ 1 within 7 days despite proper management.
3. Any other safety signal that, in the judgment of the DSMB, represents an unacceptable risk to participants.

3.1 SUBJECT'S TREATMENT AFTER THEIR PARTICIPATION IN THE STUDY

No specific treatment is planned for these patients after their participation in the study.

8. STUDY ASSESSMENTS AND PROCEDURES

A schedule of treatment phases and assessments is outlined in Table 8. It may be necessary to perform these procedures at unscheduled time points if deemed clinically necessary by the investigator

ARI-HER2 phase I clinical trial <u>Schedule of events</u>	Evaluation		Treatment period							Follow-up (FUP) up to 3 months							FUP up to 12 months						FUP up to 24 months	EOT	
	First visit (D-42) ¹	Screening (D-13 to -7) ²	D-6, -5, -4	D-3 to -1	D0 / 1 st dose	D1-2	D3 / 2 nd dose	D4-6	D7 / 3 rd dose	D10	D14	D21	D28	D42	D56	D70	D84	M4, M5	M6	M7, M8	M9	M10, M11	M12	Every 3 months	End of treatment (at PD)
													±1	±1	±1	±3	±3	±7	±7	±7	±10	±10	±10	±14	
STUDY PROCEDURES																									
Eligibility	x																								
Informed consent	x																								
Medical history ³	x																								
Anamnesis	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Physical examination ⁴	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Vital signs ⁵	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
ECOG PS	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Electrocardiogram	x	x			x				x		x		x		x		x		x		x		x	x	
Concomitant medication	Continuous from main consent																								
Adverse Events	Continuous from main consent																								
Inpatient monitoring			Daily every 4 hours (including SO ₂)																						
Body CT scan	x											x					x		x		x		x	x	
Brain MRI scan ⁶	x											x					x		x		x		x	x	
Bone scintigraphy ⁷	x											x					x		x		x		x	x	
Echocardiography	x											x					x					x	X (every 6 months)		
Serial bone densitometry assessment	x																x			x		x			
Lymphoapheresis	x ¹																								
Lymphodepleting CT ⁸			x																						
ARI-HER2 infusion ⁹					x		x		x																
LABORATORY PARAMETERS (LOCAL LAB)																									
Pregnancy test ¹⁰	x	x										x			x		x			x		x		x	
Serology ¹¹	x																								

HBV DNA ¹²	X											X		X		X		X		X		X	X	
Hematology ¹³	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Biochemistry ¹⁴	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Ferritin		X			X				X		X		X		X		X		X		X	X		X

ARI-HER2 phase I clinical trial <u>Schedule of events</u>	Evaluation		Treatment period							Follow-up (FUP) up to 3 months								FUP up to 12 months						FUP up to 24 months	EOT	
	First visit (D-42) ¹	Screening (D-13 to -7) ²	D-6, -5, -4	D-3 to -1	D0 / 1 st dose	D1-2	D3 / 2 nd dose	D4-6	D7 / 3 rd dose	D10	D14	D21	D28	D42	D56	D70	D84	M4, M5	M6	M7, M8	M9	M10, M11	M12	Every 3 months	End of treatment (at PD)	
													±1	±1	±1	±3	±3	±7	±7	±7	±10	±10	±10	±14		
TSH, free T4		x			x				x		x		x		x		x		x		x		x	x		
Urine sediment and ionogram	x	x			x				x		x		x		x		x		x		x		x	x		
CEA, CA15-3 ¹⁵		x											x				x		x		x		x	x		
High-sensitivity troponin and NT-proBNP.		x							x		x		x		x		x	x	x	x	x	x	x	x	x	
LABORATORY PARAMETERS (TO SEND PERIODICALLY TO CENTRAL LAB AT HCB)																										
Biopsy tumor sample ¹⁶	x												x												x	
Blood sample for CAR monitoring (flow cytometry and/or quantitative PCR) ¹⁷					x		x		x		x		x		x		x	x	x	x	x	x	x	x	x	
Blood sample for translational research, including ctDNA and cytokine assessment		x			x		x		x		x		x				x								x	

Table 8. Visits and procedures calendar

1. Lymphoapheresis will be performed within 14 days following the first visit, after confirmation of HER2 overexpression in a tumor sample. Apheresis products will be sent to the central laboratory at HCB for ARI-HER2 manufacturing, which will take an estimated time of 3 weeks, including quality controls.
2. A second assessment for inclusion will be performed after completion of ARI-HER2 manufacturing, a maximum of 42 days after the first visit, ≤7 days before initiating lymphodepleting CT.
3. Demographic information includes age, sex, and self-reported race/ethnicity. Reproductive status and smoking history should also be captured. Medical history includes clinically significant diseases, relevant family history (oncological) and drug allergies/intolerances.
4. A complete physical examination should include neurological exam.
5. Vital signs will include blood pressure, pulse rate, temperature and SO_2 .
6. A baseline brain MRI scan will be required (subsequently only in case of known CNS disease or neurologic symptoms).
7. Only required in case of known or suspected bone disease.
8. On days -6, -5, and -4: cyclophosphamide 300mg/m²/day + fludarabine 30mg/m²/day. If contraindicated: bendamustine 100mg/m²/day on days -5 and -4.
9. The complete dose of ARI-HER2 will be administered in three fractions (10%, 30%, and 60%), with an interval of >24h between fractions based on clinical tolerance (D0, D3, D7).
10. Only for fertile women (<60 years old). If positive/unconclusive urine test, confirmatory blood test will be performed.
11. Viral serologies including cytomegalovirus (CMV), human-immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), and Epstein-Barr virus (EBV). Viral serologies should be repeated if clinically indicated or if a positive result is obtained in the baseline test.
12. In patients with negative HbsAg but positive anti-core antibodies, HBV DNA will be tested. If negative, the patient will be candidate for the study, but HBV DNA monitoring will be required.
13. Hematology will include hemoglobin, hematocrit, RBC, WBC, MCV, MCH and MCHC. Coagulation tests will be required within 2 weeks before tumor biopsies. Except in this situation, coagulation will not need to be tested routinely, and tests will be performed according to individual clinical criteria.
14. Biochemistry will include glucose, creatinine, glomerular filtrate, transaminases (ALT/AST), gamma-glutamyl transferase, total bilirubin, alkaline phosphatase, LDH, amylase, lipase, creatine kinase, total protein and albumin count, sodium, potassium, calcium, magnesium, phosphorus, and C-reactive protein.
15. CEA: carcinoembryonic antigen; CA15-3: carbohydrate 15-3, as breast cancer tumor markers.
16. Baseline tumor biopsy will be required for screening to confirm HER2 overexpression (IHC 3+). Additional biopsies at first reevaluation (D28 ±1) and EOT will be offered to each patient (not mandatory).
17. Fresh samples will be sent in real time for flow cytometry to central laboratory. After two negative consecutive determinations, no samples will be required (only monitoring by qPCR).

The procedures expected to be performed during the different phases of the study are detailed below.

8.1 EVALUATION PHASE

I. First visit (days -42 to -13).

- The inclusion and exclusion criteria (see section 5) will be reviewed
- Obtaining informed consent
- A tumor sample obtained through a core biopsy* is required for confirmation of HER2 overexpression before screening.
- Clinical evaluation: anamnesis and record of medical history (including demographics, menopausal status, breast cancer medical history, tumor histology, prior lines of tumor treatment and response to prior lines, history of previous or present toxicities related to previous tumor treatments), concomitant medications, physical examination (including vital signs with SO_2), ECOG performance status, cardiac evaluation (by electrocardiogram and echocardiography).
- Laboratory test: Blood/urine pregnancy test (in fertile women) and viral serologies (CMV, HIV, HBV, HCV, EBV, and HBV DNA if required), hematology and biochemistry (Table 9), urine sediment and ionogram.

Table 9. Laboratory test

Blood tests	Specific parameters to be determined
Hematology	Hemoglobin (Hb) Hematocrit (Hct) Red blood cell (RBC) count White blood cell (WBC) co Mean cellular volume (MCV) Mean cellular hemoglo (MCH) Mean cellular hemoglobin concentration (MCHC)
Biochemistry	Glucose Creatinine Glomerular filtrate Alanine-aminotransferase (ALT) Aspartate-aminotransferase (AST) Gamma-glutamyl transferase (GGT) Alkal phosphatase (ALP) Total bilirubin Lactate dehydrogenase (LDH) Amylase Lipase Creatine kinase Total protein and albumin count Sodium Potassium Calcium Magnesium Phosphorus C-reactive protein

*NOTE: A coagulation test will be performed within 2 weeks before tumor biopsies. Except in this situation, coagulation tests will not be required routinely and will be performed according to individual clinical criteria for each patient.

Determination of infectious markers required by current regulations (annex III RD 9/2014 and directive 2006/17/CE) is mandatory before biopsy collection. As a minimum, the following tests will be required: human immunodeficiency virus (HIV), Hepatitis B, Hepatitis C, syphilis and HTLV-1.

Test	Acceptance criteria
Anti-HIV 1/2 Ab (MEIA), serum	Non-reactive or negative
Anti-hepatitis C Ab (MEIA), serum	Non-reactive or negative
Hepatitis B virus surface antigen	Non-reactive or negative
IgG hepatitis B virus core antigen	Negative/positive if $4 > 10$ UI/L *
RPR (Lues-RPR Ab, serum)/VDRL	Negative / positive if IgM pallidum negative
Anti-HTLV I/II Ab	Non-reactive or negative
Anti-Trypanosoma Cruzi Ab (Chagas)**	Non-reactive or negative

* In case of HBc positive / HBsAg negative, the performance of additional tests to assess the risk of product acceptance may be discussed with the MM.

**when coming from endemic areas

- Radiological examinations: a body CT scan, a brain MRI scan, and a bone scintigraphy (in cases of known or suspected bone involvement) will be performed during the screening phase.

II. Lymphoapheresis (days -42 to -28)

Lymphoapheresis will be performed within the 14 days following the first visit and after confirmation of HER2 overexpression in a tumor sample.

Blood samples will be sent to the central laboratory at HCB for ARI-HER2 manufacturing, which will take an estimated time of 3 weeks, including quality controls.

III. Screening visit (days -13 to -7)

Assessment for inclusion will be performed after the completion of ARI-HER2 manufacturing, a maximum of 42 days after the first visit, and ≤ 7 days before CART- cells product infusion at day 0. Procedures will include:

- Clinical evaluation: anamnesis, complete physical examination (including height, weight and vital signs with SO_2), ECOG performance status, electrocardiogram, concomitant medication and assessment of AEs/SAEs.
- Laboratory test; Blood/urine pregnancy test (in fertile women), hematology, biochemistry, ferritin and thyroid profile determination, urine sediment and ionogram, and tumor markers (CEA, CA15-3).
- Blood samples collection for translational research.

8.2 TREATMENT PHASE

I. Lymphodepleting CT (Days -6 to -1)

Patients will be admitted to the in-patient ward and lymphodepleting chemotherapy will be administered (cyclophosphamide $300\text{mg}/\text{m}^2/\text{day}$ + fludarabine $30\text{mg}/\text{m}^2/\text{day}$ on days -6, -5, and -4; if contraindicated: bendamustine $100\text{mg}/\text{m}^2/\text{day}$ on days -5 and -4).

Patients will be closely monitored (daily every 4 hours), including anamnesis, physical

examination, weight, vital signs with SO₂, ECOG performance status, concomitant medication and SAEs. Blood/urine samples will be collected daily to perform hematology and biochemistry determination, in addition to other studies that may be clinically indicated.

II. ARI-HER2 infusion (Days 0 to 7)

ARI-HER2 infusion will be delivered in 3 fractions within this period (on days 0, 3 and 7).

Patients will remain admitted and closely monitored, including:

- Clinical evaluation: daily anamnesis and physical examination, vital signs with SO₂, weight (on days 0 and 7), ECOG performance status, electrocardiogram (on days 0 and 7), concomitant medication and assessment of AEs/SAEs.
- Laboratory test; Blood/urine pregnancy test (in fertile women), hematology, biochemistry, ferritin and thyroid profile determination (only on days 0 and 7), urine sediment and ionogram (only on days 0 and 7).
- Blood samples collection on days 0, 3 and 7 for cytokine induction assessment, ARI-HER2 monitoring through cytometry and quantitative PCR and translational research.

8.2.1 Follow-up (FUP) phase (from day 10 to end of treatment)

I. FUP up to 3 months [days 10, 14, 21, 28 (+/-1), 42 (+/-1), 56 (+/-1), 70 (+/-3) and 84 (+/-3)]

Procedures will include:

- Clinical evaluation; anamnesis, physical examination, vital signs with SO₂, weight, assessment of ECOG performance status, and assessment of concomitant medication and SAEs in all visits. Electrocardiograms will be performed on the visits corresponding to days 14, 28, 56 and 84.
- Radiological examinations: body CT scan +/- brain CT/MRI scan in case of known central nervous system involvement, bone scintigraphy in case of known bone disease will be performed around the day 28 visit.
- Laboratory test: Blood/urine pregnancy test (in fertile women, only on day 28, 56 and 84), hematology, biochemistry, ferritin and thyroid profile determination (only on days 14, 28, 56 and 84), urine sediment and ionogram (only on days 14, 28, 56 and 84), and tumor markers (CEA, CA15-3, only on days 28 and 84).
- Blood samples: collection on days 14, 28, 56 and 84, and sent to the central laboratory for cytokine induction assessment, ctDNA monitoring and translational research, and ARI-HER2 monitoring through cytometry and quantitative PCR.
- Optional biopsy: an optional biopsy will be offered to patients on day 28 visit.

II. FUP up to 24 months (monthly from month 4 to month 12, then every 3 months up to month 24)

Procedures will include:

- Clinical evaluation: anamnesis, physical examination, vital signs with SO₂, weight, assessment of ECOG performance status, and assessment of concomitant medication and SAEs in all visits. Electrocardiograms will be performed every 3 months.
- Radiological examinations: body CT scan +/- brain CT/MRI scan in case of known central nervous system involvement and bone scintigraphy in case of known bone disease will be performed every 3 months.
- Laboratory test: Blood/urine samples pregnancy test (in fertile women, every 3 months), hematology and biochemistry (all visits), ferritin and thyroid profile determination (every 3 months), urine sediment and ionogram (every 3 months), and tumor markers (every 3 months).
- Blood samples collection: every visit for centrally ARI-HER2 monitoring (through cytometry and quantitative PCR).

8.2.2 End of treatment (EOT) visit

Procedures will include anamnesis, physical examination, vital signs with SO₂, weight,

assessment of ECOG performance status, and assessment of concomitant medication and SAEs. Blood samples will be collected for hematology, biochemistry, and ferritin determination. Additional blood samples will be collected for translational research and ARI-HER2 centrally monitoring through cytometry and quantitative PCR. Optional biopsy: an optional biopsy will be offered to patients on the EOT visit.

8.3 MULTICENTER COORDINATION AND PROCEDURES STANDARDIZATION

To ensure consistency, safety, and scientific integrity in this trial, coordinated procedures and centralized oversight are essential. Given the complexity and risk profile of ARI-HER2 administration, the study will work with harmonized operational standards across all participating sites when feasible. The following measures will be used to establish a framework for synchronized patient management, uniform application of eligibility criteria, standardized site training, centralized data and laboratory assessments, and robust safety monitoring.

1. Sentinel patient at the coordinating center: the first patient of the trial will be treated at HCB, and expansion to the other hospitals will occur after DSMB review of preliminary safety data.
2. Central slot management: a global calendar managed by the Medical Monitor will guarantee that only one patient at a time is treated across all centers, ensuring the observation period established between patients.
3. Central eligibility confirmation at two time points: each patient's eligibility will be reviewed by the Medical Monitor before apheresis and before lymphodepletion to guarantee consistent application of criteria.
4. Standardized site training: before site activation, each team (PI, ICU, on-call physicians, pharmacy, apheresis, data managers) will receive central training regarding trial design, toxicity management and crucial procedures.
5. Centralized safety monitoring and data integrity: a single electronic CRF, centralized data management, and harmonized monitoring procedures will guarantee data consistency across sites. Rapid SAE reporting (within 24 hours), weekly safety calls during ARI-HER2 administration, and DSMB review after each cohort will ensure coordinated oversight of the product safety profile.
6. Central laboratory assessments: ARI-HER2 persistence, cytokine monitoring, and biopsy review will be performed centrally at HCB to ensure uniform methodology.

These measures, combined with centralized IMP manufacturing and controlled staggered dosing, provide the homogeneity of procedures, coordinated timing, and safety oversight expected for a first-in-human dose-finding study, while enabling timely accrual.

9 TRANSLATIONAL RESEARCH AND BIOLOGICAL SPECIMENS

Translational research will be performed by specialized scientists of Translational Genomics and Targeted Therapeutics in Solid Tumors Lab (IDIBAPS) in future studies. These studies will be performed using tumor biopsy and blood samples only if the patient consents to store. Samples will be stored within the collection of Biospecimens of patients with Solid Tumors at the Hospital Clinic led by Dr. Aleix Prat (ID number: R160630-176). Samples from the infusion product will be stored at the Immunology department, and two aliquots will be sent to Dr. Prat's lab for translational research.

9.1 TUMOR TISSUE SAMPLES

A tumor biopsy will be performed during the screening phase to confirm HER2 overexpression. Additional optional biopsies will be offered to patients on the visit corresponding to the first evaluation (day +28) and end of treatment (EOT) to assess the

presence of ARI-HER2 in the tumor site and correlate histological and molecular studies with clinical outcomes.

9.2 BLOOD SAMPLES

Blood samples will be collected at different timepoints (before lymphodepleting chemotherapy, before ARI-HER2 infusion, 3 and 7 days after infusion, and periodically during the follow-up period). Blood will be collected aseptically by venipuncture into sterile tubes. These samples will be used for ARI-HER2 monitoring through cytometry and quantitative PCR, for cytokine induction assessment, and for translational research, including ctDNA determination.

9.3 SAMPLE STORAGE AND DESTRUCTION

Any blood or tumor samples collected according to the Table of Assessments can be analyzed for any of the tests outlined in the protocol. All residual samples (or leftover biologic samples after protocol-defined studies are completed) obtained during the study (FFPE, fresh-frozen, plasma, etc.) will be stored, if the patient consents, in an academic central repository. The repository will be Biospecimens of patients with solid tumors led by Dr. Aleix Prat (ID number: R160630-176). The specimens in the study repository will be made available for future biomarker research towards further understanding of treatment combination, of breast cancer, related diseases, and adverse events, and the development of potential, associated diagnostic assays.

The subject retains the right to request that the sample material be destroyed by contacting the investigator. Following the request from the subject, the investigator is to provide the sponsor with the required study and subject number so that any remaining blood or tumor samples and any other components from the cells can be located and destroyed. The FCRB is the exclusive owner of any data, discoveries, or derivative materials from the sample materials and is responsible for the destruction of the sample(s) at the request of the subject through the investigator, at the end of the storage period, or as appropriate (i.e. the scientific rationale for experimentation with a certain sample type no longer justifies keeping the sample).

10 SAFETY ASSESSMENT

Primary and secondary safety endpoints are described in sections 4.1 and 4.2 respectively.

10.1 DEFINITIONS

Adverse event (AE)

An AE is any untoward medical occurrence in a clinical study subject administered a medicinal (investigational or non-investigational) product. An AE does not necessarily have a causal relationship with the treatment. An AE can therefore be any unfavourable and unintended sign (including an abnormal finding), symptom, or disease temporally associated with the use of a medicinal (investigational or non-investigational) product, whether related to that medicinal (investigational or non-investigational) product or not (Definition per International Conference on Harmonization [ICH]).

This includes any occurrence that is new in onset or aggravated in severity or frequency from the baseline condition, or abnormal results of diagnostic procedures, including laboratory test abnormalities.

Adverse Event of Special Interest (AESI)

An AESI is a pre-specified medically significant event that has the potential to be causally

associated with ARI CAR T-cell treatment and that needs to be carefully monitored.

Serious adverse event (SAE)

A SAE is any untoward medical occurrence that at any dose: results in death, is life-threatening, requires inpatient hospitalization or prolongation of existing hospitalization, results in persistent or significant disability/incapacity, is a congenital anomaly/birth defect, is a suspected transmission of any infectious agent via a medicinal product, is medically important (according to the treating physician), other important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the subject or may require intervention to prevent one of the other outcomes listed in the definition above.

Any event requiring hospitalization (or prolongation of hospitalization) that occurs during the course of a subject's participation in the study (from the ICF signing during the screening period to the completion of the follow-up phase at 24 months from ARI-HER2 treatment) must be reported as an SAE, except for the following: hospitalizations not intended to treat an acute illness or AE (eg, social reasons such as pending placement in long-term care facility), surgery or procedure planned before entry into the study.

Adverse reaction (AR)

An AR is an AE suspected to be causally related to a medicinal product.

An AR is a response to a medicinal product which is noxious and unintended. Response in this context means that a causal relationship between a medicinal product and an adverse event is at least a reasonable possibility (i.e. the relationship cannot be ruled out). Adverse reactions may arise from use of the product within or outside the terms of the marketing authorization or from occupational exposure. Conditions of use outside the marketing authorization include overdose, misuse, abuse and medication errors. [Sources EMA GVP Annex I, and ICH E2A].

Unexpected Adverse reaction (UAR)

Any adverse reaction, whose nature or severity of which is not consistent with the applicable product information (i.e. investigator's brochure for an unauthorized investigational product or summary of product characteristics for an authorized product).

Suspected Unexpected Serious Adverse Reaction (SUSAR)

A SUSAR is an adverse reaction that is both serious and unexpected.

10.2 ASSESSMENT OF INTENSITY

An assessment of intensity grade will be made using the general categorical descriptors outlined in the CTCAE version 5.0 (see Appendix XXX). The investigator should use clinical judgment in assessing the severity of events not directly experienced by the subject (i.e. laboratory abnormalities).

10.3 ASSESSMENT OF CAUSALITY

-AE related: The relationship in time of the AE with the study drug indicates a possible causal relationship and it cannot be explained by factors such as the patient's clinical condition or therapeutic interventions.

-AE unrelated: The relationship in time of the AE with the study drug indicates an unlikely causal relationship, or other factors (concomitant medication or conditions) or other therapeutic interventions provide a satisfactory explanation for the AE.

10.4 COLLECTION AND FOLLOW UP OF ADVERSE EVENTS

Clinical records of patients must include detailed information of all adverse events detected by care providers and/or communicated by patients. Given the seriousness of the pathology suffered by participants, not all of adverse events will be recorded in the Case Report Form (CRF). Adverse events that may not be recorded in the CRF are those events that are

specific to the patient's pathology and those that are present at the time of inclusion, unless there is a worsening of these events during participation in the study and/or they are clinically relevant adverse events. On the other hand, any SAE, AESI or any adverse event not a priori attributable to the underlying disease needs to be recorded in the case report form.

For all recordable adverse events information regarding the event, MedDra codification, start and end dates, intensity, action taken, causal relation and outcome will be registered.

10.4.1 Follow up of non-serious adverse events

For all AEs, the investigator must pursue and obtain information adequate both to determine the outcome of the AE and to assess whether it meets criteria for classification as a serious adverse event (SAE) requiring immediate notification (see section 7.4.2). Follow-up of the AE is required until the event resolves or stabilizes at a level acceptable to the investigator. The investigator must follow all the AE and update the information in clinical records and, if it's considered a recordable event, in the study CRF too.

10.4.2 Procedures for immediate reporting of serious adverse events by the investigator to the sponsor

Any serious adverse event (SAE) must be reported immediately to SOLT I to be discussed with the sponsor/authorization holder in 24 hours from the moment in which investigator has knowledge of the event, regardless of its relationship to the investigational drug. Notification of SAE will be carried out through the serious adverse event form included in the case report form and must be sent at the address included in the SAE template. The investigator must provide with all the documentation related to the event (additional laboratory tests, discharge reports, etc ...) registered in the SAE form. In addition, the investigator will respond to any request for tracking information or any questions the sponsor may ask in relation to the SAE/SAR, in the same terms as for the initial communication. The person in charge pharmacovigilance at SOLT I will inform the HCB-QPPV about all SAEs that may arise in a regular basis. Regarding SAE, a follow-up report may be necessary until the event resolves or stabilizes.

10.4.3 Procedures for immediate reporting of unexpected serious adverse reactions by the sponsor to Competent Authorities

Any suspicion of a serious adverse reaction (SUSAR) must be notified to competent authorities through the Eudravigilance platform within a maximum period of 15 calendar days, 7 calendar days in the event of serious and unexpected fatal or life-threatening adverse reactions, all in compliance with Royal Decree 1090/2015 and Regulation (EU) No. 536/2014 of the European Parliament and of the Council, of April 16 2014.

The information on adverse events that are not serious or unexpected and those that are considered recordable and not related to the treatments under study will be collected in a tabulated form at the end of the clinical trial or coinciding with the interim analyses when these were foreseen.

SOLT I will maintain detailed records of all SAEs that are communicated to it by the researchers. These records will be presented to the AEMPS when it so requests.

10.5 DOSE-LIMITING TOXICITIES

The dose-limiting toxicities (DLTs) are detailed below:

- Grade ≥ 3 cytokine-release syndrome (CRS).
- Grade ≥ 2 immune-effector cell-associated neurotoxicity syndrome (ICANS).
- Grade ≥ 2 immune-effector cell-associated hemophagocytic-like syndrome (IEC-HS).
- Grade ≥ 2 pneumonitis or myocardiopathy attributable to CAR-related damage of HER2-expressing cells from healthy tissues.

- Any other grade ≥ 3 on-target off-tumor toxicity (including diarrhea, altered liver enzymes, and others) without clinical improvement within 48-72 hours following last dose administration.
- Any other severe life-threatening adverse event attributable to ARI-HER2 infusion, not including complications derived from AEs related to pre-conditioning lymphodepleting chemotherapy.

A 3+3 dose-escalation design will be used to determine the maximum tolerated dose (MTD) (see section 12.4).

11 ASSESSMENT OF EFFICACY

11.1 SPECIFICATION OF THE EFFICACY PARAMETERS

Secondary efficacy endpoints are described in section 3. The parameters to assess ARI-HER2 efficacy will be:

- Clinical Benefit Rate (CBR): Percentage of patients who have a complete response (CR), partial response (PR), stable disease (SD) or non-PR/non-progressive disease (Non-PD) lasting >24 weeks, based on local investigator's assessment according to iRECIST criteria. Radiological assessments will be performed at month 1 of ARI-HER2 administration and subsequently every 3 months until progressive disease or end of study.
- Objective response rate (ORR): Percentage of patients with $\geq 30\%$ reduction (PR) and/or tumor disappearance (CR) after treatment as determined by the iRECIST criteria. Radiological assessments will be performed at month 1 of ARI-HER2 administration and subsequently every 3 months until progressive disease or the end of the study.
- Duration of response (DOR): Time since confirmation of CR or PR after treatment with ARI-HER2, until disease progression or death (whichever occurs first). Patients alive without progression will be censored at the time of last follow-up.
- Progression-free survival (PFS): Time from administration of the ARI-HER2 product to disease progression or death (whichever is earlier). Patients alive without progression will be censored at the time of last follow-up.
- Overall survival (OS): Time between administration of the ARI-HER2 product and death of the patient from any cause. Alive patients will be censored at the time of last follow-up.

11.2 TIMING FOR ASSESSING, RECORDING, AND ANALYSING OF EFFICACY PARAMETERS

Thoraco-abdominal CT scan will be performed at 1 month after ARI-HER2 administration, and every 3 months thereafter until the end of the study (2 years of follow-up). Radiological response will be evaluated according to the iRECIST criteria. In case of bone or central nervous system (CNS) involvement, bone scintigraphy and/or brain magnetic resonance image (MRI) will also be performed, respectively.

Table 8 shows calendar and procedures needed to assess the safety and efficacy parameters.

12 STATISTICAL ANALYSIS

This is a first in human, dose-escalation, non-randomized, multicenter, clinical trial to evaluate the safety and tolerability of ARI-HER2 in patients with HER2-positive metastatic breast cancer relapsed or refractory to approved treatments.

A summary of the general statistical analysis methods is provided below; full details will be

provided in a separate Statistical Analysis Plan (SAP), which will be finalized prior to database lock of the analysis.

12.1 ANALYSIS POPULATION

The populations used for analysis will include the following:

- **Intent-to-treat (ITT) population:** This population includes all patients enrolled into the study regardless of whether they are later found to be ineligible, a protocol violator, never treated or not evaluated. Patients for whom the production of the investigational product has been confirmed and the inclusion criteria reviewed will be included in the ITT population.
- **Safety population:** This group includes all patients who receive lymphodepleting chemotherapy (see section 5.1).
- **DLT evaluable population:** For purposes of dose escalation decisions, all patients who complete the DLT period will be included in this population. Patients who receive supportive care during the DLT assessment window that confounds the evaluation of DLTs may be excluded from this population. Patients who do not complete the DLT assessment due to the observation of a DLT will be included.
- **Response evaluable population:** This population comprises all patients who receive the study treatment and had at least one follow-up assessment for efficacy. Patients with clinical progression before the first assessment for efficacy will be considered as a non-responder.

12.2 DATA ANALYSIS

Baseline characteristics

Baseline characteristics will be summarized for the safety population. For categorical variables, numbers (with percentages) will be provided. For continuous variables, means (with standard deviations), or medians (with range) will be presented.

Safety analysis

Descriptive statistics will summarize the frequency and severity of adverse events and treatment-limiting toxicities. Time to onset and duration of these events will be reported. The incidence of DLTs will be used to determine the MTD and RP2D.

Adverse events (AEs) will be reported i) overall and ii) for each dose level and presented as tables of frequency of AEs and by worse severity grade. Separate tabulations will be produced for all treatment emergent AEs (TEAEs), treatment-related AEs, SAEs, discontinuations due to AEs, and AEs of at least Grade 3 severity. By-subject listings will be provided for deaths, SAEs, DLTs, and AEs leading to discontinuation of treatment.

Efficacy Analysis

For categorical efficacy endpoints (ORR), counts and percentages, with 95% Clopper-Pearson confidence interval, will be calculated. Time-to-event endpoints (PFS, DoR and OS) will be analyzed according to the Kaplan-Meier method. Kaplan-Meier survival curves and the median estimation (50th percentiles) will be reported. In addition, rates at specific timepoints can also be estimated. If necessary, as an exploratory analysis, univariate Cox proportional hazard models will be used to calculate the risk reduction. Hazard ratio (HR) with 95% CIs will be reported to evaluate difference between subgroups of interest. If needed, Kaplan-Meier curves will be estimated for subgroups of interest.

Exploratory Analysis

Exploratory data analysis techniques can be used to identify potential biomarkers and perform subgroup analyses to explore differential effects. Different translational research studies will be performed in the future (see section 9.0). Longitudinal models such as mixed-effects models may be used to analyze this data.

12.3 SAMPLE SIZE

No formal sample size calculation has been performed for this study, as no hypothesis testing is being evaluated. A classical 3+3 design will be used for the dose escalation process. This study is designed to potentially evaluate 3 cohorts (dose levels strategies, see section 7.2) and at least 3 patients need to be treated at each dose level. The sample size for this study will range between 3 and 18 patients. The final number will depend on the dose-escalation process and the DLTs observed in the first cohort. However, it is expected to evaluate 9-15 patients. The sample size will be underpowered to detect specific differences in efficacy endpoints, but it should allow for preliminary observations of the clinical activity.

12.4 DOSE-ESCALATION PROCESS

This trial will use a 3+3 design for the dose-escalation process. Using this design, three patients will be initially enrolled in cohort 1 and treated with the initial dose of 1.5×10^6 cell/Kg (see section 7.2). The dose-escalation rules will be as follows:

- If no DLTs are observed in the first three patients, the dose under investigation will be considered safe at that level. Consequently, the trial will escalate to the next higher dose level, and a new cohort of three patients will be treated at this increased dose.
- If one DLT is observed in the first three patients, an additional cohort of three patients will be enrolled and treated at the same dose level to further evaluate the safety profile of that dose level. If no additional DLTs are observed among these six patients, the trial will escalate to the next higher dose level. However, if one or more additional DLTs are observed (making it two or more in total), the dose level will be considered too toxic.
- If two or more patients in a cohort experience DLTs, this indicates that the current dose level is too toxic. In this case, no further patients will be treated at this dose level, and the previous lower dose level will be considered the MTD.

12.5 PROCEDURES OF HANDLING MISSING, AND SPURIOUS DATA

All available efficacy and safety data will be included in data listings and tabulations. No imputation of values for missing data will be performed unless otherwise stated. The relevance of missing sample data will be assessed.

12.6 STUDY SAFETY STOPPING CRITERIA

The study may be terminated at any time and for any reason at the discretion of the Sponsor relating to observed safety or clinical efficacy parameters (see section 7.8). The study team will conduct meetings after evaluating 3 patients in each cohort to evaluate safety and efficacy data and make decisions on the study's follow-up (see section 13.13).

12.7 PATIENT FLOW THROUGH TRIAL

Patient flow through the trial will be described including the number of patients entered into and evaluable in each cohort and the number of patients who discontinued along with the reasons for discontinuation. This will be presented in a tabular format.

A CONSORT flow diagram will be used to summarize the number of patients who were:

- Assessed for eligibility at screening (number of patients eligible and ineligible at screening, and reasons for ineligibility).
- Eligible at screening but not included in the study and specific reasons.
- Included in the study by each dose level.
- Number of patients who were not correctly treated (and reasons).
- Included in the dose-escalation population by each dose level.

13 PRACTICAL ISSUES

13.1 DIRECT ACCESS TO SOURCE DOCUMENTS/DATA

13.1.1 Data management. Electronic Case Report Form (eCRF)

The electronic Case Report Form (e-CRF) will be designed by sponsor or delegate to record, according to their instructions, all observations, and other data pertinent to the Study. All e-CRFs should be completed in their entirety in a neat, legible manner to ensure accurate interpretation of data. Should a correction be made, the corrected information will be entered in the e-CRF overwriting the initial information. An audit trail allows identifying the modification. Additional details will be described in the Data Management Plan.

13.1.2 Software

The software used to monitor this clinical study will be REDCAP v.14, an open-source software for Electronic Data Capture (EDC) developed by Vanderbilt University for Clinical Data Management (CDM).

13.1.3 Design of forms

For its design, an annotated CRF on word format will be prepared, which will be agreed on with the sponsor. Once the variables to be collected at each of the visits and the modules of the CRF have been agreed, the DM will program it.

13.1.4 Enter data

Data entry will be performed through SOLT's REDCAP web platform. All records of the Subjects enrolled in the Study will be accessible via the web platform by the relevant roles from the start of the Study to the database closure.

The process for data entry via the web will be detailed in the Data Entry User Manual. Data will be available to the sponsor as soon as they are entered in the e-CRF system. The computerized handling of the data when available in the e-CRF may generate additional requests (DRF) to which the Investigator is obliged to respond by confirming or modifying the data questioned. The requests along with their responses will be managed through the e-CRF.

13.1.5 Responsibilities

It is the responsibility of the Investigator to maintain adequate and accurate eCRF completion, which for this trial will be of electronic nature.

Data are available to the Sponsor as soon as they are entered in the eCRF system. The computerized handling of the data in the eCRF may generate additional requests which the Investigator is obliged to respond by confirming or modifying the data questioned. The requests along with their responses will be managed through

13.2 RECORDING OF DATA

Procedures shall be employed, and controls designed to ensure the confidentiality of electronic records. Such procedures and controls must include validation of systems to ensure accuracy and reliability, ability to generate accurate and complete copies of records, protection of records to enable retrieval, use of secure, computer-generated, time-stamped entries, use of operational system checks, use of device checks to determine validity of source data input, determination that those who develop, maintain, or use such systems have adequate education and training, the establishment and adherence of written policies to deter record falsification, the use of appropriate controls over systems documentation including the distribution or use of documentation for system operation and maintenance, and revision and change control procedures, which document time-sequenced development and modifications of systems documentation.

13.2.1 Direct access to source data/documents

To ensure compliance with the Clinical Trial Protocol, GCP, and applicable regulatory requirements, the Investigator should permit auditing by or on the behalf of the Sponsor and

by regulatory authorities.

The Investigator agrees to allow the auditors/inspectors to have direct access to his/her study records for review, being understood that these personnel is bound by professional secrecy, and, as such, will not disclose any personal identity or personal medical information.

The Investigator will make every effort to help with the performance of the audits and inspections, giving access to all necessary facilities, data, and documents. As soon as the Investigator is notified of a planned inspection by the authorities, he/she will inform the Sponsor and authorize the Sponsor to participate in this inspection. The confidentiality of the verified data and the protection of the patients should be respected during these inspections. Any results and information arising from the inspections by the regulatory authorities will be immediately communicated by the Investigator to the Sponsor.

The Investigator must take appropriate measures required by the Sponsor in relation to corrective actions for all problems found during the audit or inspections.

13.2.2 Archiving and storage of data

The investigator is responsible for maintaining all records which enable the conduct of the clinical trial at the site to be fully documented, in compliance with ICH GCP filing standard. Timeliness and completeness of the documentation will be regularly checked by the clinical monitor. The documentation of the clinical trial including all the relevant correspondence should be kept by the investigator for the minimum period required by applicable regulatory and country-specific requirements.

All completed study related documents (e.g. eCRF, Informed consent forms, drug accountability logs, staff signature lists, Subject identification log, ...) must be archived for 25 years after the finalization of the study.

13.2.3 Audit and inspection

The investigators agree to comply with the requirements of the sponsor and the competent authority for an auditor and/or inspection of the study.

The audit can apply to all stages of the study, from the development of the protocol to publication of the results and filing the data used or produced in the study.

13.3 QUALITY CONTROL AND QUALITY ASSURANCE

According to ICH/GCP guidelines, the sponsor should ensure that the trial is adequately monitored.

The purpose of monitoring is to verify that the rights and wellbeing of human subjects are protected; that the trial is accurate, complete and verifiable with source data and that the trial is conducted in compliance with the protocol, GCP and the applicable regulatory requirements.

The monitoring plan will establish the guideline for conducting all the monitoring activities. To ensure homogeneity and the same quality standards, monitors in all study sites will be trained with the same procedures. The training sessions will be done before the initiation of the trial and training periodic updates if new sites or personnel are included or when overall quality of data collected through the eCRF is not optimal. The training session will be focused on the protocol implementation, target population and recruitment, electronic CRF and site initiation visit (SIV) procedures and monitoring forms to be used along the study conduct.

During the SIV, the monitors will train the local investigators and collaborators about study protocol, requirements, interventions, visits, follow-up activities and electronic CRF completion. These tasks will ensure the quality of the data and the quality of the conduct of the clinical trial.

At regular intervals during the Clinical Trial the site will be contacted through monitoring visits, letters, or telephone calls by a representative of the Monitoring Team to review study progress, Investigator and patient compliance with Clinical Trial Protocol requirements, and any emergent problems.

The Monitor will visit the study site on a regular basis to ensure that the study is conducted and documented in accordance with this protocol, ICH GCP guidelines, regulatory requirements, and any study specific documents such as eCRF completion guidelines.

Monitoring visits will be conducted to confirm that:

- The investigational team is adhering to the study protocol.
- IC has been obtained from all patients.
- AEs have been reported as required.
- Data are being accurately recorded on the eCRFs.
- IMP is being stored correctly, and drug accountability is being performed on an on-going basis.
- Facilities are, and remain, acceptable throughout the study.
- The Investigator and the site are receiving sufficient training, information, and support throughout the study.

Moreover, during monitoring visits, the data recorded on the eCRFs, source documents, and other study-related records will be compared against each other in order to ensure accurate data that reflect the actual existence of the subject in the study, i.e., source data verification.

Detailed monitoring visit information will be provided separately in the Monitoring Plan, it will be approved before the first patient is included.

13.4 ETHICAL AND REGULATORY CONSIDERATIONS

This study will be conducted in accordance with the protocol and ethical principles stated in the Declaration of Helsinki or the applicable guidelines on GCP, and all applicable local laws, rules, and regulations:

- Regulation 536/2017 of the European Parliament of 16 April 2014 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice (GCP) in the conduct of clinical trials on medicinal products for human use.
- Royal Decree 1090/2015, of 4 December regulating clinical trials with medicinal products, Ethics Committees for investigation with medicinal products (IRB/EC) and the Spanish Clinical Studies Registry, that introduced substantial amendments in national legislation so as to make feasible the application of Regulation (EU) No 536/2014 of the European Parliament and of the Council, and to develop those aspects that the Regulation leaves to national legislation.
- Guidelines on Good Clinical Practice specific to Advanced Therapy Medicinal Products.
- Spanish Law 9/2003, of 25 April, establishing the legal regime for the contained use, voluntary release and placing on the market of genetically modified organisms and by Royal Decree 178/2004 of 30 January, that introduced substantial amendments in national legislation so as to make feasible the application of these law.

13.5 ETHICS APPROVAL

The Protocol, informed consent form, Investigator's Brochure and other required documents must be approved by the Ethics Committee before enrollment of subjects in the study. The letter of approval from the Ethics Committee, as well as a list of documents reviewed, will be filed in the Investigator Site File (ISF) and a copy will be filed in the trial master file (TMF) held by the Sponsor.

The Sponsor and his delegates, in collaboration with the investigator, will be responsible for reporting to the Ethics Committee all changes in research activity, including protocol amendments, updates of Investigator's Brochures, annual safety reports, all unanticipated problems involving risks to human subjects, and study termination.

13.6 REGULATORY CONSIDERATIONS

In parallel to the submission to the Ethics Committee, the Sponsor has to obtain an authorisation from the appropriate competent authority to conduct the clinical study. Subjects must not be entered into the study until the relevant Ethics Committee has issued its opinion and the CA has given authorisation to conduct the study.

All substantial amendments must be submitted to the Ethics Committee and/or to the Competent authorities for approval.

Neither the investigator nor the CRO will modify this protocol without a formal request from the sponsor. All protocol amendments must be issued by the sponsor and signed and dated by the investigator. Protocol amendments must not be implemented without prior Ethics Committee approval (if applicable i.e. IMPD-exclusive substantial amendments), or when the relevant competent authority has raised any grounds for non-acceptance, except when necessary to eliminate immediate hazards to the subjects, in which case the amendment must be promptly submitted to the Ethics Committee and relevant competent authority.

Documentation of amendment approval by the investigator and Ethics Committee must be provided to the sponsor. During the study, in situations where a deviation from the protocol is unavoidable, the investigator or other physician in attendance will contact the appropriate sponsor representative. Except in emergency situations, this contact should be made before implementing any deviation from the protocol. The data recorded in the eCRF and source documents will reflect any deviation from the protocol (protocol breaches), and the source documents will describe this departure and the circumstances requiring it. All protocol deviations will be recorded by the *Protocol Deviation Log*. In the event that a major deviation and/or a *Serious Breach* related to the study protocol—but not limited to it—is identified, the monitoring team will notify the Sponsor or its representatives. The Sponsor or its representatives will assess the seriousness of the event and determine the appropriate course of action. Once the severity has been established, the monitoring team will be informed. If a *Serious Breach* is confirmed, it will be notified within a maximum of seven (7) days from its detection, as stipulated by applicable regulations.

All information related to protocol deviations will be further detailed and reflected in the *Protocol Deviation Plan*.

13.7 RESPONSIBILITY OF INVESTIGATORS

The investigator is responsible for ensuring that the study is performed in accordance with the protocol, current ICH guidelines on Good Clinical Practice (GCP), and applicable regulatory and country-specific requirements. Good Clinical Practice is an international ethical and scientific quality standard for designing, conducting, recording, and reporting studies that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety, and well-being of study subjects are protected, consistent with the principles that originated in the Declaration of Helsinki (and updated according to its last version, Fortaleza, Brazil, 2013), and that the study data are credible.

In addition, the investigator is responsible for giving information about the study to all staff members involved in the study or in any element of subject management, before and during the study.

The investigator is also responsible for ensuring the privacy, health, and welfare of the subjects during and after the study. The investigator(s) must be familiar with the background and requirements of the study and with the properties of the investigational product as described in the Investigator's Brochure.

Before the start of the study, the investigator (or sponsor where required) will provide the Ethics Committee with current and complete copies of the following documents (as required by local regulations).

13.8 INFORMED CONSENT

Each subject must give written consent according to local requirements after the nature of

the study has been fully explained. The ICF(s) must be signed before performance of any study related activity. The ICF(s) that is/are used must be approved by both the sponsor and by the reviewing Ethics Committee and be in a language that the patient can read and understand.

The informed consent should be in accordance with principles that originated in the Declaration of Helsinki (updated according to its last version, Fortaleza, Brazil, 2013), current ICH and GCP guidelines, applicable regulatory and country-specific requirements, and sponsor policy. Before enrollment in the study, the investigator or an authorized member of the study site personnel must explain to potential subjects the aims, methods, reasonably anticipated benefits, and potential hazards of the study, and any discomfort that the participation in the study may entail.

Subjects will be informed that their participation is voluntary and that they may withdraw consent to participate at any time. They will be informed that choosing not to participate will not affect the care the subject will receive for the treatment of his or her disease. Subjects will be told that alternative treatments are available if they refuse to take part and that such refusal will not prejudice future treatment. Finally, they will be told that the investigator will maintain a subject identification register and that their records may be accessed by health authorities and authorized sponsor personnel without violating the confidentiality of the subject, to the extent permitted by the applicable law(s) or regulations. By signing the ICF the subject is authorizing such access, including permission to obtain information about his or her survival status, and agrees to allow his or her study physician to recontact the subject for the purpose of obtaining consent for additional safety evaluations, if needed, and subsequent disease-related treatments, or to obtain information about his or her survival status.

The subject will be given sufficient time to read the ICF and the opportunity to ask questions. After this explanation and before entry into the study, consent should be appropriately recorded by means of the subject's personally dated signature. After having obtained the consent, a copy of the ICF must be given to the subject.

13.9 PATIENT CONFIDENTIALITY

The collection and processing of personal data from subjects enrolled in this study will be limited to those data that are necessary to fulfil the objectives of the study. Patient data will be anonymized to ensure confidentiality and compliance with applicable data privacy protection laws and regulations.

Subjects will be codified with a study code that prevents their identity from being deduced. The PI and duly authorized collaborators will commit to maintain personal data strictly confidential, according to the corresponding country-specific requirements. The link between the numeric code and real personal data from subjects will be rigorously kept by the PI. The informed consent obtained from the subject includes explicit consent for the processing of personal data and for the investigator/institution to allow direct access to his or her original medical records (source data/documents) for study-related monitoring, audit, Ethics Committee review, and regulatory inspection.

In the case report form, the patient will only be identified by the assigned study code. The name of patients will not appear in any publication or report of the study results.

The participation of the patient in the trial will be noted in their medical records.

The investigator will complete a list which will include the names of the patients participating in the trial, the study inclusion number, and their medical history. Only investigators and the staff responsible for guaranteeing data quality and data analysis will have access to the clinical documentation of the participants.

Duly authorized persons by the sponsor and the health authorities and the Ethics Committee may audit or inspect the trial. Personal information will not be publicly available, in compliance with General Data Protection Regulation (EU GPDR 2016/679 of 27th April). In addition, the sponsor cannot make decisions as to the significance of any findings resulting from exploratory research. Therefore, exploratory research data will not be returned to subjects or investigators, unless required by law or local regulations. Privacy

and confidentiality of data generated in the future on stored samples will be protected by the same standards applicable to all other clinical data.

Only data collected for the study that does not bear any information that could directly identify the patient will be transferred to third parties or other countries. Should this transfer occur, it will be for the same purposes as the study and guarantee confidentiality with at least the level of protection afforded by applicable regulations in Spain.

Patients will be informed that their clinical data will be incorporated into an automated study specific file and the results of the clinical trials and different studies conducted with samples can be communicated at scientific meetings, medical conferences or publications.

However, patient's identity or identifiable data will never be disclosed.

13.10 PERSONAL DATA PROTECTION

The processing, communication and transfer of personal data of all participants will be adjusted to compliance with Regulation EU 2016/679 of the European Parliament and the Council of 27 April 2016 on the protection of natural persons as to the processing of personal data and the free circulation of data, being mandatory as of May 25, 2018. The legal basis that justifies the processing of your data is the consent given in this act, in accordance with the provisions of the Article 9 of the EU Regulation 2016/679.

The data collected for the study will be collected only identified by a code, so that no information will be included to identify the participants. Data will be processed with the only purpose of carry out all activities related to the clinical trial in compliance with pharmacovigilance regulations (for the drug safety control). The legal basis for the processing of the data is the participant consent and Article 9.2 of the Regulation. Only the study doctor and his collaborators have the right to access the source data (clinical history) and will be able to relate the data collected in the study with the patient's medical history. The identity of the participants will not be available to any other person except for a medical emergency or legal requirement. Health authorities, the Research Ethics Committee and personnel authorized by the Sponsor of the study, may have access to the personal data identified when necessary to verify data and study procedures, but always maintaining confidentiality in accordance with current legislation.

Only encrypted data will be transferred to third parties and to other countries, which in no case will contain information that can identify the participant directly (such as name and surnames, initials, address, social security number, etc.). If this assignment occurred, it would be for the same purpose of the study described and guaranteeing confidentiality.

If a transfer of encrypted data is carried out outside the EU, either in entities related to the hospital where the patient participates, to service providers or to researchers who collaborate with them, the data of the participants will be protected by safeguards such as contracts or other mechanisms established by the data protection authorities.

The sponsor of the trial commits to carry out the data processing according to EU Regulation 2016/679 and, therefore, to keep a record of the processing activities to carry out and to make a risk assessment of the data processing, to establish what measures will be applied and how it will be done.

In addition to the rights already covered by the previous legislation (access, modification, opposition and cancellation of data, deletion in the new Regulation) participants can now limit the processing of data collected for the project that has to be rectified, request a copy or move to a third party (portability). To exercise these rights, the participant should be directed to the principal investigator of the study or the Data Protection Delegate of their site. The participant also has the right to contact the Data Protection Agency if not satisfied. The data cannot be deleted even if a patient discontinues the study, to guarantee the validity of the investigation and comply with the legal duties and the medication authorization requirements.

The Investigator and the Sponsor are obliged to keep the data collected for the study at least up to 30 years after its completion. Subsequently, personal information will only be kept by the site for the care of their health and by the sponsor for other scientific research purposes if the patient has given their consent to do so, and if the law and applicable ethical

requirements so permit.

In accordance with the provisions of recital 33 of the regulations and the corresponding provisions of each country involved in the study regulations, the data may be preserved in such a way that the clinical data are kept separate from the identifiers, to be used in future investigations, applying all technical precautions necessary to avoid their re-identification, and in accordance with all ethical and legal requirements.

13.11 DATA MANAGEMENT AND RECORDS ARCHIVING

Data management and handling will be conducted according to the study specific Data Management Plan in accordance with ICH guidelines and applicable standard operating procedures, which will be prepared and approved before the first patient is included.

Data validation will be managed by the data management team and/or its designate. The data will be subjected to validation according to sponsor standard operating procedures or its designate in order to ensure accuracy in the collected eCRF data.

Before database closure, reconciliation will be performed between the SAEs entered in the safety database and the study database. After database closure, the database will be exported as data sets in a format that allow proper data analysis.

13.12 FINANCING AND INSURANCE

This study has obtained funding through an “Asociación Española Contra el Cáncer”- AECC call addressed to grant clinical research projects (code ECAEC235235DELG).

The Sponsor or its delegate will procure insurance for this clinical trial to cover trial related injuries of the participants according to local regulatory requirements.

Clinical trial participants will be provided on request with the conditions of insurance along with the patient information and consent form.

13.13 STUDY COMMITTEES

13.13.1 Data Safety Monitoring Board (DSMB)

The Data Safety Monitoring Board (DSMB) is a group of experts that monitors the main safety and tolerability outcome measures and the overall conduct of the trial with the aim of protecting the safety and interests of the trial participants.

The membership, frequency, method and the aspects to be reviewed, will be specified in the DSMB-Charter.

Each time treatment of 3 patients at the same dose level is completed, the DSMB members will meet to discuss the safety data of the cohort, and they will decide whether to proceed with dose escalation according to the 3+3 design. Additionally, the DSMB may be convened whenever safety concerns arise. Procedures related to DSMB's activity and functions will be guided by international guidelines for DSM committees.

The DSMB Chairperson will be responsible for providing a written report of findings and recommendations to the sponsor in a timely manner. The sponsor will be responsible for informing the study sites and appropriate regulatory authorities of any DSMB recommendation relating to conduct of the study. If the sponsor decides not to fully implement the advice of the DSMB, the sponsor will send the advice to the competent authorities (IRB/EC and AEMPS), including a note to substantiate why (part of) the advice of the DSMB will not be followed.

13.13.2 Follow-up Committee

A Follow-up Committee (FUC) will be created comprising investigators associated with the design or conduct of the study, as well as independent investigators. Candidates to be part of the FUC will be proposed by the Principal Investigator and the Sponsor of the study.

The FUC shall ensure that the management of the study is carried out in line with the Protocol and GCP guidelines. The FUC may propose and must review and approve any necessary amendment to the Protocol. It will also make decisions regarding the publications generated from the study data. Information on members, responsibilities and frequency of

meetings will be specified in the FUC charter.

The FUC may be consulted for advice when needed, either in face-to-face meetings or via teleconference.

13.14 PUBLICATION

The sponsor of the CT expressly agrees to publish the results of the study detailed above after the complete analysis of data.

The study will be registered in a public and accessible clinical database prior to the inclusion of the first patient.

The investigators will publish the study results in internationally indexed journals. Authorship will include the members of the study Follow-up committee which include study coordinators.

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16 APPENDICES

16.1 Appendix 1

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