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Titolo tesi

HER2 IN SOFT TISSUE SARCOMAS: A NOVEL POTENTIAL THERAPEUTIC TARGET

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SUMMARY

CHAPTER 1 - INTRODUCTION

Soft Tissue Sarcomas: an unmet clinical need

Soft tissue sarcomas (STS) represent a rare and highly heterogeneous group of malignant tumors arising in the mesenchymal tissues, accounting for less than 1% of all adult solid malignancies but encompassing more than 80 distinct histological subtypes according to the most recent World Health Organization (WHO) classification[1,2]. Despite their relative rarity, STS constitute a major clinical challenge due to their biological diversity, diagnostic complexity, and limited therapeutic options, particularly in the advanced or metastatic setting.

The annual incidence of adult-type STS in Europe is estimated at approximately 4–5 cases per 100,000 individuals, with liposarcomas and leiomyosarcomas being the most frequently diagnosed subtypes, while the majority of histologies occur at an incidence below 2 per 1,000,000 per year [3]. STS can arise in virtually any anatomical site, most commonly in the extremities, trunk, retroperitoneum, and visceral locations, often presenting as deep-seated masses with nonspecific symptoms. As a result, delayed diagnosis is common, and a substantial proportion of patients present with locally advanced or metastatic disease [4].

From a pathological standpoint, STS represent one of the most complex areas in oncologic diagnostics. Accurate classification requires the integration of morphology, immunohistochemistry, and molecular genetics, and diagnostic discordance rates of up to 20–30% have been reported outside specialized referral centers [2]. This intrinsic complexity reflects the remarkable biological heterogeneity of STS, which range from genetically simple, translocation-driven tumors to highly complex sarcomas characterized by extensive chromosomal instability and copy number alterations.

Surgery remains the cornerstone of treatment for localized disease and, when performed in high-volume reference centers, can achieve long-term disease control in a significant proportion of patients [4]. However, despite optimal local management, up to 40% of patients eventually develop metastatic disease, which is associated with a poor prognosis and a median overall survival of approximately 18–20 months [5]. In this setting, systemic therapy options remain limited. Anthracycline-based chemotherapy has represented the standard first-line treatment for decades, yielding modest response rates and a median progression-free survival of approximately six months, with no clear overall survival benefit demonstrated by combination regimens in unselected populations [5,6].

Although recent advances in molecular profiling have improved the biological characterization of sarcomas and led to histotype-specific therapeutic opportunities in a limited number of rare subtypes, most STS remain largely refractory to targeted approaches[6,7]. Immunotherapy has shown activity only in a limited subset of histologies, and predictive biomarkers remain poorly defined. Consequently, there is a compelling unmet need for novel therapeutic strategies capable of overcoming the intrinsic heterogeneity and therapeutic resistance of STS [6,7].

In this context, the identification of broadly expressed, biologically relevant targets that may be exploited by innovative drug platforms, such as antibody–drug conjugates, represents an attractive and rational approach. Exploring such targets may help bridge the gap between molecular characterization and effective systemic therapies, ultimately improving outcomes for patients with advanced soft tissue sarcomas.

Molecular pathways in sarcomagenesis

Sarcomagenesis is driven by a complex and heterogeneous spectrum of molecular alterations that reflect the biological diversity of soft tissue sarcomas (STS). As comprehensively reviewed by Damerell et al., sarcomas can be broadly categorized into tumors with simple karyotypes, characterized by recurrent chromosomal translocations and fusion oncogenes, and tumors with complex karyotypes, marked by extensive chromosomal instability, copy number alterations, and accumulation of multiple genetic events [8]. This dichotomy underpins fundamental differences in tumor biology, clinical behavior, and therapeutic vulnerability.

Receptor tyrosine kinases alterations and signal redundancy

Sarcomas with complex karyotypes (including leiomyosarcomas, dedifferentiated liposarcomas, undifferentiated pleomorphic sarcomas, and epithelioid sarcomas) frequently harbor alterations affecting key regulators of cell cycle control and genome integrity, such as TP53, RB1, CDKN2A, and PTEN [8]. Loss of these tumor suppressors leads to deregulated proliferation, impaired apoptotic responses, and activation of compensatory survival pathways. In parallel, aberrant activation of receptor tyrosine kinases (RTKs) and downstream signaling cascades (including PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways) is commonly observed across multiple STS subtypes, although often without a single dominant oncogenic driver.

Importantly, while several sarcoma subtypes are characterized by clear oncogenic dependencies, the successful therapeutic targeting of these drivers has remained difficult in most cases, with notable exceptions such as gastrointestinal stromal tumors[9]. Instead, sarcoma cells rely

on signaling redundancy, lineage plasticity, and dynamic interactions with the tumor microenvironment. Growth factor receptors such as PDGFR, EGFR, IGF1R, MET, and VEGFR are frequently overexpressed or activated, yet therapeutic inhibition of these pathways has generally resulted in modest clinical benefit outside of molecularly defined entities such as gastrointestinal stromal tumors. This biological context explains the historical failure of many targeted therapies when applied to unselected sarcoma populations.

Tumor microenvironment and stromal crosstalk

A key feature of sarcoma biology is the extensive crosstalk between tumor cells and the surrounding microenvironment. Sarcomagenesis and tumor evolution are strongly influenced by dynamic interactions between tumor cells and the surrounding microenvironment, including mesenchymal stem cells, cancer-associated fibroblasts, immune cells, endothelial cells, and extracellular matrix components [9]. These interactions contribute to cellular plasticity, stem-like states, therapy resistance, tumor dormancy, and the biological processes that ultimately enable tumor progression and metastatic spread **(Figure 1)**.

Recent advances in systemic therapy reflect a paradigm shift toward exploiting surface antigen expression rather than strict oncogenic dependence [7]. Antibody–drug conjugates (ADCs) exemplify this approach by leveraging receptor-mediated internalization to deliver highly potent cytotoxic payloads, even in tumors with low or heterogeneous target expression. Within this framework, receptors traditionally associated with epithelial cancers may represent therapeutically actionable targets in sarcomas, despite lacking a canonical driver role **(Figure 1)** [8]**.**

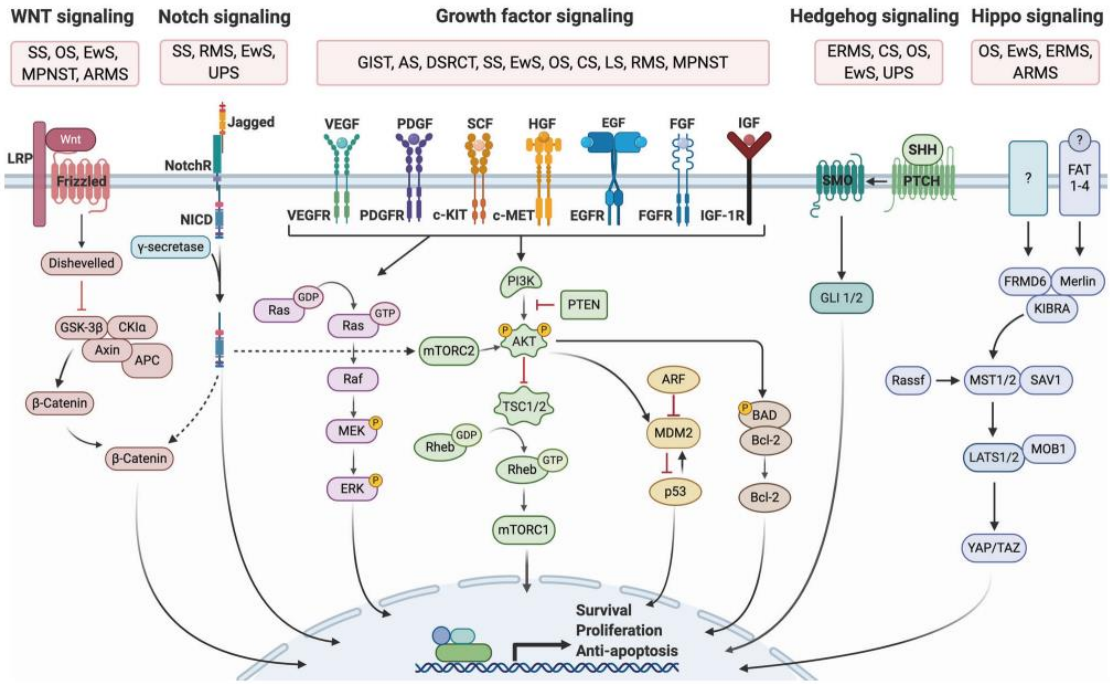

Figure 1. Molecular pathways and tumor microenvironment interactions in sarcomagenesis. Schematic overview of major signaling pathways commonly deregulated across sarcoma subtypes and their downstream effectors. **Abbreviations**: **ARMS**, alveolar rhabdomyosarcoma; **CS**, chondrosarcoma; **DSRCT**, desmoplastic small round cell tumor; **ERMS**, embryonal rhabdomyosarcoma; **EwS**, Ewing sarcoma; **GIST**, gastrointestinal stromal tumor; **LS**, leiomyosarcoma; **MPNST**, malignant peripheral nerve sheath tumor; **OS**, osteosarcoma; **RMS**, rhabdomyosarcoma; **SS**, synovial sarcoma; **UPS**, undifferentiated pleomorphic sarcoma. *Adapted from Damerell et al., 2021.*

Genomic instability and therapeutic plasticity

The evolutionary and adaptive nature of sarcomas translates into intrinsic resistance to conventional cytotoxic agents and to many single-pathway targeted therapies. Polyclonal tumor progression, the coexistence of proliferative and dormant cell populations, and sustained interactions with the tumor microenvironment collectively limit durable treatment responses. Within this framework, the exploration of alternative therapeutic strategies capable of exploiting tumor surface antigen expression, rather than strict oncogenic dependence, emerges as a rational approach to overcome biological complexity and therapeutic resistance **(Figure 2)** [9].

Taken together, the molecular landscape of STS is characterized by genomic complexity, pathway redundancy, and profound cellular plasticity. These features challenge conventional targeted therapy paradigms but simultaneously create opportunities for innovative drug platforms capable of translating biological expression into therapeutic vulnerability.

In recent years, antibody-drug conjugates (ADCs) have reshaped the therapeutic landscape of several solid tumors by enabling selective delivery of highly potent cytotoxic agents to cancer cells. Their clinical success has expanded the concept of targetability beyond classical oncogenic drivers, supporting the investigation of HER2 as a therapeutic delivery address even in tumors not primarily dependent on ERBB2 signaling[10].

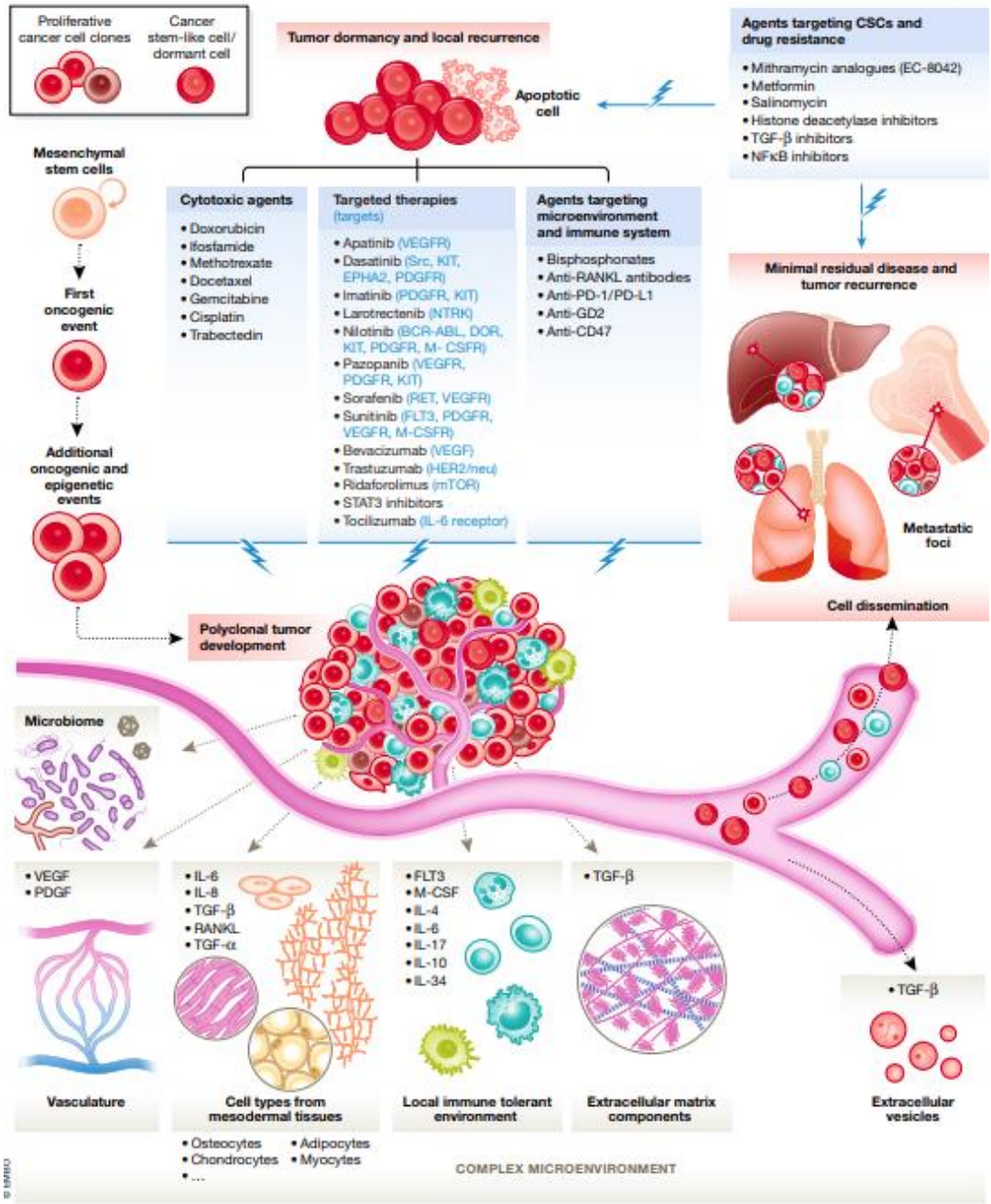

Figure 2. Therapeutic challenges and tumor evolution in sarcomas. Schematic representation of sarcoma evolution from early oncogenic events to polyclonal tumor development, highlighting tumor dormancy, dissemination, and recurrence. The figure

summarizes major therapeutic classes and illustrates how microenvironmental components may contribute to drug resistance and minimal residual disease. **Abbreviations**: **CSCs**, cancer stem cells; **VEGF/VEGFR**, vascular endothelial growth factor/(receptor); **PDGF/PDGFR**, platelet-derived growth factor/(receptor); **TGF-β**, transforming growth factor beta; **IL-6**, interleukin-6; **RANKL**, receptor activator of NF-κB ligand; **PD-1/PD-L1**, programmed cell death 1/(ligand 1); **NF-κB**, nuclear factor kappa B; **mTOR**, mechanistic target of rapamycin; **NTRK**, neurotrophic tyrosine receptor kinase; **FLT3**, Fms-like tyrosine kinase 3; **M-CSF**, macrophage colony-stimulating factor. A*dapted from Grunewald et al., 2020.*

CHAPTER 2 - HER2 BIOLOGY AND THERAPEUTIC TARGETING

2.1 HER2 (ERBB2): Structure and Physiological Role

Human epidermal growth factor receptor 2 (HER2), also known as ERBB2, is a member of the ErbB family of receptor tyrosine kinases, which also includes EGFR (HER1), HER3, and HER4 [11,12]. Structurally, HER2 is a 185-kDa transmembrane glycoprotein composed of an extracellular ligand-binding domain, a single-pass transmembrane region, and an intracellular domain with intrinsic tyrosine kinase activity. Unlike other ErbB family members, HER2 has no known activating ligand and exists in a constitutively open conformation, rendering it the preferred dimerization partner within the family[12–14]. This constitutively open conformation facilitates rapid heterodimer formation with other ligand-activated ErbB receptors, particularly HER3, thereby amplifying downstream signaling output.

Physiological activation of HER2 signaling occurs through homo- or, more commonly, heterodimerization with other ErbB receptors following ligand binding to the partner receptor. Among these, the HER2/HER3 heterodimer is considered the most potent signaling unit, due to the multiple binding sites of HER3 for the p85 subunit of PI3K.[12,13] Dimerization leads to autophosphorylation of intracellular tyrosine residues and subsequent activation of key downstream pathways, including the PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, and JAK/STAT cascades, ultimately regulating cell proliferation, survival, differentiation, and motility[11,12].

At the structural level, recent advances in crystallography and cryo-electron microscopy have provided detailed insights into the conformational dynamics of HER2. The extracellular domain displays a constitutively exposed dimerization arm, explaining the high propensity of HER2 to engage in heterodimer formation **(Figure 3)**[13,14]. These structural features are central to both physiological signaling and oncogenic activation, and they also critically influence the binding and activity of HER2-targeted therapeutics[14].

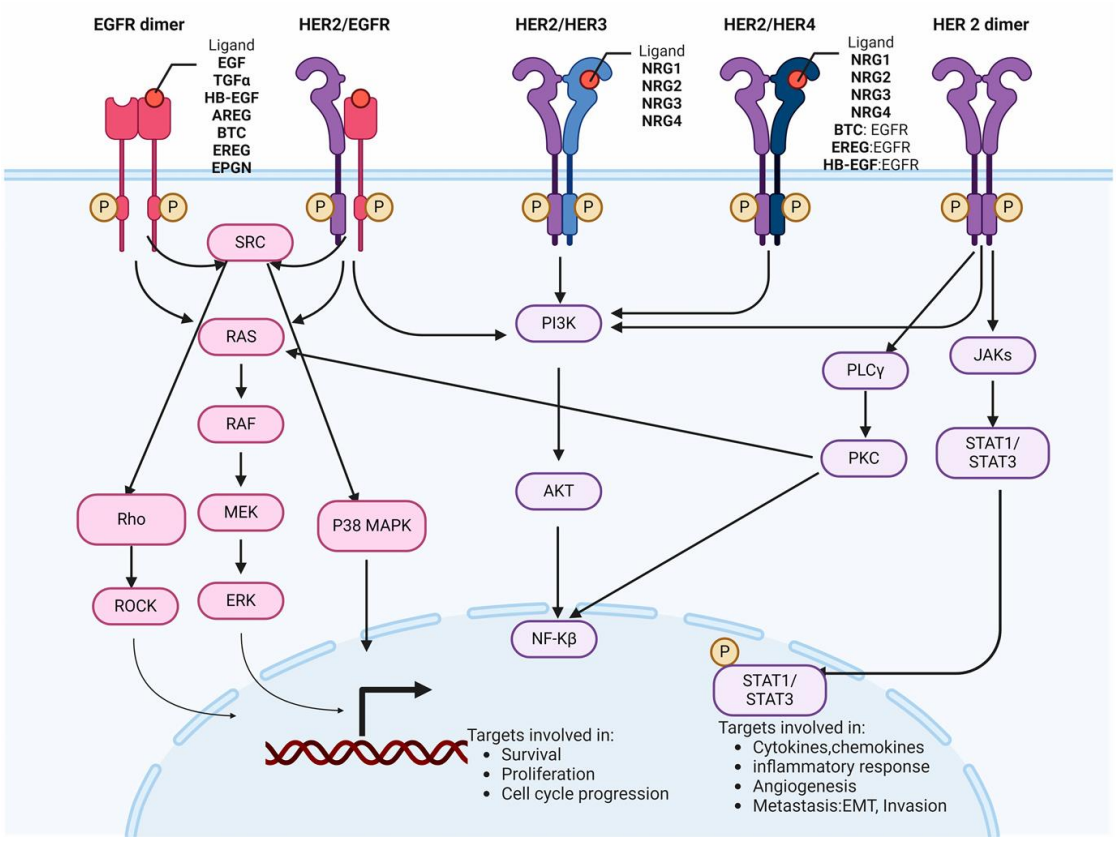

Figure 3. Schematic representation of HER2 signaling regulation. HER2 is the preferred partner for forming heterodimers (pairing) with other EGFR family members, such as HER1 (EGFR) or HER3. HER2 is the preferred partner for forming heterodimers (pairing) with other EGFR family members, such as HER1 (EGFR), HER3, and HER4. When a ligand binds to other EGFR family members, dimerization occurs activating the intrinsic tyrosine kinase activity of HER2, resulting in initiating downstream signaling cascades. These pathways contribute to regulating gene expression, cell cycle progression, cell motility, and other cellular functions. Figure was made using BioRender. and HER4. When a ligand binds to other EGFR family members, dimerization occurs, which activate the intrinsic tyrosine kinase activity of HER2, resulting in initiating downstream signaling cascades. These pathways contribute to regulating gene expression, cell cycle progression, cell motility, and other cellular functions. **Abbreviations: EGF**, epidermal growth factor; **TGFα**, transforming growth factor alpha; **HB-EGF**, heparin-binding EGF-like growth factor; **AREG**, amphiregulin; **BTC**, betacellulin; **EREG**, epiregulin; **EPGN**, epigen; **NRG1-4**, neuregulins 1-4; **PLCγ**, phospholipase C gamma; **PKC**, protein kinase C; **JAK**, Janus kinase; **STAT**, signal transducer and activator of transcription; **NF-κB**, nuclear factor kappa B; **EMT**, epithelial-mesenchymal transition. A*dapted from Cheng, 2024.*

2.2 HER2 as an Oncogenic Driver

Dysregulation of HER2 signaling represents a well-established oncogenic mechanism across multiple human cancers. In breast and gastric cancers, HER2-driven tumorigenesis is primarily associated with ERBB2 gene amplification, leading to marked protein overexpression and ligand-independent receptor activation[11].

Beyond amplification and overexpression, activating somatic mutations of ERBB2 have been identified in a variety of solid tumors, including lung, biliary, cervical, and colorectal cancers[15]. These mutations, frequently located within the kinase or transmembrane domains, can induce constitutive HER2 signaling and oncogenic transformation, although their sensitivity to HER2-targeted therapies varies according to tumor type and molecular context[12,15].

This biological heterogeneity has led to the conceptual distinction between "HER2-addicted tumors", in which tumor growth is critically dependent on HER2 signaling, and "HER2-expressing tumors", in which HER2 is present but does not function as the dominant oncogenic driver[15,16]. While classical HER2-directed therapies have demonstrated profound clinical benefit in HER2-addicted malignancies, their efficacy has been inconsistent in tumors where HER2 expression is low, heterogeneous, or biologically secondary[16].

2.3 Assessment of HER2 status

Accurate determination of HER2 status is essential for therapeutic decision-making and has traditionally relied on immunohistochemistry (IHC) and in situ hybridization (ISH) techniques[17]. IHC assesses HER2 protein expression at the tumor cell membrane and is scored on a semi-quantitative scale from 0 to 3+, whereas ISH evaluates ERBB2 gene amplification. In breast cancer, American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) guidelines define HER2 positivity as IHC 3+ or IHC 2+ with confirmed gene amplification[17].

However, these criteria were developed in the context of HER2-addicted breast cancer and may not be fully applicable to other tumor types. HER2 expression can be heterogeneous, dynamic, and context-dependent, and significant inter-assay and inter-observer variability has been reported[16,17]. Traditionally, HER2 status has been conceptualized as a binary variable, categorizing tumors as either HER2-positive or HER2-negative based on protein overexpression or gene amplification. However, increasing evidence supports the existence of a continuous spectrum of HER2 expression, ranging from complete absence of detectable protein to high-level

overexpression. Recent pathological frameworks have formalized this concept by defining intermediate categories such as HER2-low, typically corresponding to immunohistochemical scores of 1+ or 2+ without ERBB2 amplification, and HER2-ultra-low, characterized by faint or incomplete membranous staining in a limited fraction of tumor cells **(Figure 4)**[18].

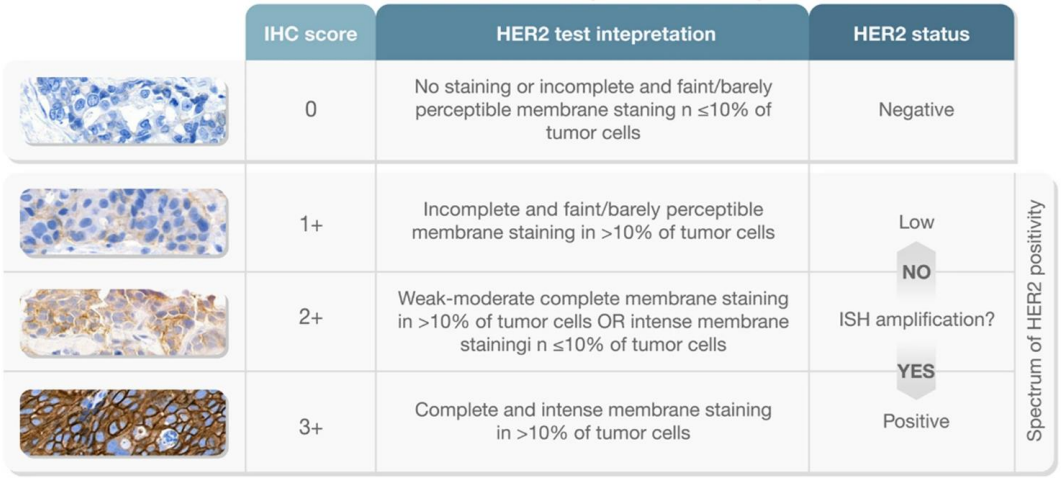

Figure 4. Spectrum of HER2 positivity according to ASCO/CAP guidelines. Comprehensive visual representation of HER2 expression levels in BC depicting the final HER2 status through pathological interpretation and scoring. IHC, immunohistochemistry; ISH, in situ hybridization. Breast Biomarker Reporting, CAP Cancer Protocol Templates. **Abbreviations: HER2**, human epidermal growth factor receptor 2; **IHC**, immunohistochemistry; **ISH**, in situ hybridization.

Importantly, these categories do not represent distinct biological subtypes but rather reflect quantitative and spatial variations in HER2 protein expression. The clinical relevance of this spectrum has emerged with the advent of antibody–drug conjugates, whose mechanism of action relies on receptor-mediated internalization rather than oncogenic addiction. In this context, even low-density or heterogeneous HER2 expression may be sufficient to confer therapeutic vulnerability, redefining HER2 from a binary biomarker into a continuous, functionally exploitable target.

A critical and often underappreciated challenge in HER2 assessment is the variable analytical performance of available anti-HER2 antibody assays at the lower end of the expression spectrum. While assays have been optimized for HER2-positive (IHC 3+) detection, their ability to reproducibly discriminate IHC 0 from IHC 1+ and IHC 2, differs substantially across platforms. Among the most widely used clones, HercepTest (polyclonal A0485/DG44), Ventana PATHWAY 4B5, and SP3 exhibit measurable differences in sensitivity at low expression levels: using calibrated receptor-density bead standards, HercepTest detects HER2 at significantly lower

surface concentrations than 4B5, classifying a larger proportion of cases as HER2-low (35% vs. 19%; $p<0.01$), whereas 4B5 demonstrates higher specificity and fewer equivocal IHC 2+ results. A quantitative inter-laboratory comparison across 35 centers confirmed that SP3 showed the lowest sensitivity for low-level detection, while 4B5 OptiView and DG44 exhibited the highest sensitivity and lowest inter-laboratory variability[19,20]. Pre-analytical variables, such as cold ischemia time, fixation duration, antigen retrieval conditions, and section thickness, further influence staining intensity, with effects disproportionately amplified at the low expression range where signal-to-noise discrimination is narrow. Inter-observer reproducibility is also substantially lower at the HER2-low threshold than for IHC 3+ scoring. Focused pathologist training and external quality assurance participation improve but do not eliminate this discordance. These limitations are particularly relevant in the sarcoma setting, where HER2 expression is predominantly low-density and assessed without validated, histotype-specific scoring criteria, amplifying the risk of both false-negative and false-positive classification. This underscores the need for sarcoma-adapted HER2 testing protocols before HER2-directed ADC strategies can be reliably applied in this context[21].

2.4 HER2-targeted therapeutic strategies

HER2-targeted therapies encompass several pharmacological classes, including monoclonal antibodies (mAbs), tyrosine kinase inhibitors (TKIs), and antibody-drug conjugates (ADCs)[11,13,15]. Monoclonal antibodies such as trastuzumab and pertuzumab bind distinct extracellular epitopes of HER2, inhibiting receptor dimerization, promoting receptor internalization, and inducing antibody-dependent cellular cytotoxicity[11,16]. TKIs, including lapatinib and neratinib, target the intracellular kinase domain and block downstream signaling pathways[15].

Despite their transformative impact in HER2-addicted breast cancer, both mAbs and TKIs have shown limited efficacy in many HER2-expressing tumors, largely due to pathway redundancy, compensatory signaling, and intrinsic or acquired resistance mechanisms[12,15,16]. In contrast, ADCs represent a paradigm shift by decoupling therapeutic efficacy from strict oncogene addiction. By exploiting HER2 as a surface antigen for receptor-mediated internalization, ADCs deliver highly potent cytotoxic payloads directly into tumor cells, even when HER2 expression is low or heterogeneous[13,16].

This property redefines HER2 from a purely oncogenic driver to a functionally exploitable therapeutic target. Consequently, HER2-directed ADCs provide a strong biological rationale for

exploring HER2-targeted strategies in tumor types traditionally considered HER2-negative, including malignancies of mesenchymal origin such as soft tissue sarcomas[13,16].

CHAPTER 3 - HER2 IN SARCOMAS: STATE OF THE ART

The evaluation of HER2 expression in soft tissue sarcomas represents a complex and evolving field characterized by significant methodological challenges, conflicting evidence, and emerging therapeutic opportunities. While HER2 has been established as a clinically actionable target in breast and gastric cancers, its role in sarcomas remains incompletely understood, shaped by decades of inconsistent findings and failed therapeutic attempts. A pivotal tissue microarray analysis of 37,992 tumors reported zero HER2 IHC 3+ cases among 1,211 soft tissue sarcomas, indicating that "breast- like" HER2 overexpression is exceptionally rare when stringent, epithelial- cancer criteria are applied[22]. However, the advent of next-generation antibody-drug conjugates (ADCs) and tumor-agnostic approvals has renewed interest in HER2 as a potential therapeutic vulnerability in select sarcoma subtypes.

Recent comprehensive querying of TCGA Pan Cancer Atlas and ICGC/TCGA datasets via cBioPortal has provided systematic, unbiased assessment of ERBB2/HER2 alterations across 289 soft tissue sarcoma samples, revealing striking histotype-specific heterogeneity. In Cohort 1 (TCGA Pan Cancer Atlas, n=255 sarcomas), ERBB2 overexpression (defined by copy number amplification and/or elevated mRNA) was detected in:

- Synovial sarcoma: 50% (5/10) [highest]
- MPNST: 44% (4/9)
- Myxofibrosarcoma: 4% (1/25)
- Undifferentiated pleomorphic sarcoma (UPS): 2% (1/50)
- Dedifferentiated liposarcoma (DDLPS): 2% (1/59)
- Leiomyosarcoma (LMS): 1% (1/100)

Notably, Cohort 2 (ICGC/TCGA Nature 2020 study, n=34 sarcomas comprising 19 DDLPS and 15 LMS) demonstrated zero ERBB2 alterations (0% amplification, mRNA elevation, or protein overexpression), emphasizing the rarity of HER2 changes in these specific histotypes.

3.1 Historical Evidence of HER2 Expression in Sarcomas

The literature documenting HER2 expression in sarcomas spans more than two decades and reveals substantial heterogeneity in reported prevalence rates, largely attributable to inconsistent methodologies, variable scoring systems, and the biological diversity inherent to sarcoma histotypes[23].

Synovial sarcoma has been among the most extensively studied sarcoma subtypes with respect to HER2 expression. Early immunohistochemical studies suggested that 20-50% of synovial sarcoma specimens express HER2 protein, with some reports indicating approximately 50% overexpression[24,25]. These findings generated considerable interest in HER2 as a potential therapeutic target in this aggressive sarcoma subtype, which predominantly affects adolescents and young adults and carries limited effective treatment options beyond conventional chemotherapy. Subsequent investigations using HER2-targeted cellular immunotherapies have validated the presence of cell-surface HER2 expression in molecularly confirmed synovial sarcoma cell lines. These cell lines demonstrated high HER2 surface expression and proved susceptible to HER2-directed chimeric antigen receptor (CAR) T-cell cytotoxicity, with robust interferon-gamma and tumor necrosis factor-alpha secretion upon target recognition[26].

Osteosarcoma and Ewing sarcoma. The historical literature on HER2 in bone sarcomas is marked by profound inconsistency. Early reports documented HER2 overexpression in 32% of osteosarcoma and 16% of Ewing sarcoma biopsies[27]. Other immunohistochemical series reported HER2 positivity ranging from 32–78% in osteosarcoma, with strong membranous staining (IHC 3+) identified in 34.4% of cases in an Egyptian cohort[28]. Contrastingly, other reports showed complete absence of HER2 expression in both osteosarcoma and Ewing sarcoma using rigorous molecular techniques, directly contradicting prior reports[29]. These discrepancies highlight the impact of antibody selection, fixation protocols, and scoring criteria on reported prevalence.

Malignant peripheral nerve sheath tumors (MPNST) represent another frequently HER2-altered soft tissue sarcoma. Earlier studies identified HER2 amplification using a chromogenic in situ hybridization (CISH) in 40% (10/25) of MPNST specimens, with zero amplifications in benign neurofibromas ($p<0.001$), suggesting potential biological significance in malignant transformation[30]. However, subsequent transcriptomic analysis has complicated this interpretation: EGFR amplification (28% of MPNST cases) appears more frequent than ERBB2 amplification[31]. At the protein level, erlotinib (EGFR inhibitor) demonstrated superior anti-proliferative activity compared to trastuzumab (HER2 antibody) in MPNST cell lines, suggesting EGFR may be therapeutically more relevant[31].

Leiomyosarcoma. HER2 expression in leiomyosarcoma appears exceptionally rare across independent datasets. TCGA Cohort 1 identified ERBB2 overexpression in only 1/100 (1%) LMS cases, while Cohort 2 (which comprised exclusively LMS and DDLPS) showed 0/15 (0%) cases with any ERBB2 alteration. The molecular landscape of LMS is characterized instead by complex chromosomal rearrangements and frequent TP53/RB1 pathway alterations, with limited evidence supporting HER2 as a therapeutically actionable target[32].

DDLPS similarly demonstrates minimal HER2 expression. TCGA Cohort 1 identified ERBB2 overexpression in only 1/59 (2%) DDLPS cases; Cohort 2 showed 0/19 (0%). Comprehensive genomic profiling of 48 DDLPS specimens through Caris Life Sciences confirmed absence of HER2 expression by immunohistochemistry. DDLPS is molecularly defined by amplification of 12q13–15 containing MDM2 (74%), CDK4 (65%), and HMGA2 (29%), rather than receptor tyrosine kinase activation[33].

Myxoid liposarcoma. Limited literature specifically addresses HER2 in myxoid liposarcoma. Molecular pathogenesis is characterized by FUS-DDIT3 or EWSR1-DDIT3 fusions and immature adipogenic differentiation. Rather than HER2, myxoid liposarcoma exhibits FGFR2 overexpression, with FGFR inhibition impairing tumor growth in vitro. CXCR4 and PDGFR also show elevated expression compared to other liposarcoma subtypes[34].

Gastrointestinal stromal tumors (GIST) are driven by KIT (75–80%) or PDGFRA (10–15%) activating mutations rather than HER2 alterations. Nevertheless, HER2 expression has been detected in GIST subsets with prognostic implications. Abd El-Aziz et al. found that HER2 positivity correlated with high-risk grade ($p=0.04$), tumor size >5 cm ($p=0.001$), elevated mitotic count ($p<0.001$), and tumor relapse ($p<0.001$); particularly, 100% of relapsed GISTs (12/12) demonstrated HER2 expression versus only 10% of non-relapsed cases[35]. However, the large tissue microarray study detected zero HER2 IHC 3+ expression in 143 GIST samples, and other studies questioned whether HER2 plays a functional role in GIST pathogenesis[22,36]. This discordance suggests low-level HER2 expression may represent a biomarker of aggressive biology rather than a therapeutically actionable target.

Epithelioid sarcoma, an ultra-rare SMARCB1-deficient malignancy affecting adolescents and young adults, has not been systematically studied for HER2 expression. While EGFR is expressed in approximately 20% of bone and soft tissue neoplasms, specific HER2 data for epithelioid sarcoma are lacking[37]. The therapeutic landscape focuses on EZH2 inhibition, checkpoint blockade, and SMARCB1-loss-targeting agents, with no evidence supporting HER2 as a relevant biomarker or target.

Chordoma, a primary bone sarcoma arising from notochordal remnants, demonstrates variable HER2 expression across studies. Weinberger et al. found HER2 staining patterns in 10 primary chordomas ranging from absent (3 cases) to strong 3+ (4 cases), notably with both recurrent chordomas showing zero expression[38]. Dewaele et al. reported 11/16 chordomas as HER2-negative, 4 with low staining, and 1 with intense staining in <10% of cells; HER2 positivity was associated with EGFR co-expression[39]. Conversely, Fasig et al. found no HER2 expression in 21 chordomas. The dominant receptor tyrosine kinases in chordoma are EGFR (67%) and PDGFRB (100%), with strong EGFR–c-Met correlation[40].

As shown in **Table 1**, reported HER2 expression in sarcomas is highly heterogeneous across histologic subtypes and individual studies. The highest rates of HER2 positivity were generally reported in studies accepting both membranous and cytoplasmic staining, whereas series applying stricter membranous-only criteria, particularly in tissue microarray-based analyses and ASCO/CAP-oriented assessments, frequently found no HER2-positive cases. This discrepancy is particularly evident in osteosarcoma and GIST, for which early IHC-based studies suggested variable degrees of positivity, while later standardized analyses reported uniformly negative results. Overall, these findings suggest that the apparent prevalence of HER2 positivity in sarcomas is strongly influenced by methodological and interpretative differences.

Histotype	Samples (N)	Method	Staining Pattern Accepted	HER2+ Rate Reported (%)	Reference
Synovial Sarcoma	N=19	IHC	Membranous + Cytoplasmic	21% (IHC 2+/3+)	[24]
Synovial Sarcoma	N=19	IHC	Membranous + Cytoplasmic	~50%	[41]
Synovial Sarcoma	Cell lines (N=3)	Flow cytometry	Membranous (cell surface)	High surface expression (all 3 lines)	[26]
MPNST	N=25	FISH (DISH)	Gene amplification	40% (10/25)	[30]
MPNST	Cell lines	IHC + RT-PCR	Membranous + Cytoplasmic	Variable; ERBB2 < EGFR	[31]
Osteosarcoma	N=78 biopsies	IHC	Membranous + Cytoplasmic	32%	[27]

Histotype	Samples (N)	Method	Staining Pattern Accepted	HER2+ Rate Reported (%)	Reference
Osteosarcoma	N=64	IHC	Membranous + Cytoplasmic	32–78% (IHC 3+: 34.4%)	[28]
Osteosarcoma	N=68	IHC + FISH + RT-PCR	Membranous only	0%	[29]
Osteosarcoma	N=405 (8 studies)	Meta-analysis (IHC ± FISH)	Membranous + Cytoplasmic (pooled)	Variable (pooled)	[42]
Osteosarcoma	N=1,211 (TMA)	IHC (TMA)	Membranous only (ASCO/CAP)	0% (0/1,211)	[22]
Leiomyosarcoma	N=TMA subset	IHC (TMA)	Membranous only	0%	[22]
Dedifferentiated Liposarcoma	N=48	IHC (Caris profiling)	Not specified	0%	Jagosky 2023
GIST	N=40	IHC	Membranous + Cytoplasmic	Correlated with relapse (100% relapsed cases+)	[35]
GIST	N=143 (TMA)	IHC (TMA)	Membranous only	0% (0/143)	[22]
GIST	Multiple samples	IHC + FISH	Membranous only	Not expressed (IHC 0 predominant)	[36]
Chordoma	N=10	IHC	Membranous + Cytoplasmic	Variable: 0% in 3 cases, 3+ in 4 cases	[38]
Chordoma	N=16	IHC + FISH	Membranous + Cytoplasmic	11/16 negative; 1 intense in <10%	[39]
Chordoma	N=21	IHC	Membranous only	0% (0/21)	[40]

Table 1. Reported HER2 expression across selected sarcoma histotypes according to sample type, detection method, staining pattern considered acceptable, and HER2-positive rate. Marked inter-study variability is observed, likely reflecting differences in histology, sample source, assay platform, and interpretation criteria, particularly with regard to acceptance of membranous-only versus combined membranous/cytoplasmic staining. **Abbreviations: HER2**, human epidermal growth factor receptor 2; **IHC**, immunohistochemistry; **FISH**, fluorescence in situ hybridization; **DISH**, dual in situ hybridization; **RT-PCR**, reverse-transcription polymerase chain reaction; **TMA**, tissue microarray; **MPNST**, malignant peripheral nerve sheath tumor; **GIST**, gastrointestinal stromal tumor; **EGFR**, epidermal growth factor receptor; **ASCO/CAP**, American Society of Clinical Oncology/College of American Pathologists.

3.2 Membranous versus Cytoplasmic Staining: A Critical Distinction

The profound variance in reported HER2 expression rates across sarcoma subtypes reflects fundamental methodological heterogeneity. Unlike breast and gastric cancers with standardized ASCO/CAP and Hofmann guidelines respectively, no consensus HER2 assessment criteria exist for sarcomas. Studies have variably employed different primary antibodies, antigen retrieval protocols, scoring thresholds (>10–30% positive cells), and staining pattern interpretations (membranous versus cytoplasmic)[21,43].

This distinction carries mechanistic implications. In breast cancer, HER2 positivity is defined exclusively by complete circumferential membranous staining in >10% of tumor cells. Many sarcoma studies, however, have scored cytoplasmic HER2 immunoreactivity as positive. Cytoplasmic localization suggests aberrant protein trafficking and does not represent functionally active, ligand-accessible receptor amenable to antibody-based targeting[44]. The landmark tissue microarray study by Yan et al. analyzing 37,992 tumor specimens across diverse cancer types revealed that stringent HER2 IHC 3+ expression was virtually absent in non-epithelial malignancies, with 0% positivity among 1,211 soft tissue sarcomas[22]. This finding directly contradicts smaller, methodology-variable series reporting 20–50% positivity and underscores the critical importance of applying breast-cancer-standard criteria when assessing sarcoma HER2 status[28].

3.3 HER2 as a Prognostic Marker in Sarcomas: Unresolved Questions

The prognostic significance of HER2 in sarcomas remains unresolved, with conflicting data across histotypes. In osteosarcoma, HER2 positivity has been associated with adverse outcomes in some studies: a meta-analysis of 8 studies and 405 patients found HER2 overexpression correlated with worse event-free survival (HR 1.91; 95% CI 1.11–3.31; $p=0.02$) and overall survival (HR 2.51; 95% CI 1.55–4.07; $p<0.0001$)[42]. Scotlandi et al. linked HER2 to P-glycoprotein expression, suggesting a mechanistic connection between HER2 signaling and chemoresistance[27].

Paradoxically, other studies found opposite results. Tabak et al. observed that HER2 expression was down-regulated in metastatic osteosarcoma at diagnosis ($p=0.006$), proposing HER2 as a marker of favorable rather than adverse prognosis[28]. In GIST, while HER2 correlated

with relapse risk, the large tissue microarray series detected zero HER2 3+ cases, questioning whether true HER2 overexpression (by stringent criteria) is relevant to prognosis[36].

3.4 HER2-Targeted Therapy in Sarcomas: Why Trastuzumab failed

Despite suggestive preclinical and epidemiological evidence, clinical translation of HER2-targeted therapy with trastuzumab has proven disappointing. The pivotal Children's Oncology Group trial (AOST0121; NCT00023998) evaluated trastuzumab in combination with intensive multiagent chemotherapy in 96 children and adolescents with newly diagnosed metastatic osteosarcoma. Among these, 41 patients had HER2 overexpression (IHC 2+), and 34 HER2-positive patients received trastuzumab with cisplatin, doxorubicin, methotrexate, ifosfamide, and etoposide plus dexrazoxane. Results were uniformly negative: 30-month event-free survival was identical between HER2-positive and HER2-negative cohorts (32% vs. 32%; p=NS), and overall survival showed no significant difference (59% vs. 50%). While trastuzumab proved safe with anthracyclines and cardioprotection, no therapeutic benefit was attributable to HER2 blockade[45].

Preclinical work explains the potential mechanisms of failure, demonstrating that trastuzumab lacked single-agent activity against osteosarcoma or Ewing sarcoma cell lines; antitumor effects required combination with anti-IGF-IR antibodies[27]. The fundamental biological difference likely explains trastuzumab's failure: in breast and gastric cancers, HER2 positivity reflects ERBB2 gene amplification with massive receptor overexpression (hundreds of thousands per cell) and signaling addiction. In contrast, most sarcomas express HER2 without amplification at levels substantially lower than epithelial malignancies, falling below thresholds for effective antibody-dependent cellular cytotoxicity (ADCC) or receptor internalization[21].

3.5 Tumor-Agnostic Approvals and Next-Generation ADCs

ADCs and the bystander effect paradigm

Modern antibody-drug conjugates represent a transformative approach, combining HER2-targeting monoclonal antibodies with membrane-permeable cytotoxic payloads via cleavable linkers. This design enables "bystander killing" of HER2-low or HER2-negative neighboring cells, a property particularly valuable in tumors with heterogeneous HER2 expression. First-generation ADCs with non-cleavable linkers (e.g., trastuzumab emtansine/T-DM1) lack this bystander effect and require uniformly high antigen expression for efficacy[46,47].

Tumor-agnostic therapy has emerged as a transformative oncology principle. On April 5, 2024, the FDA granted accelerated approval to fam-trastuzumab deruxtecan-nxki (T-DXd;

Enhertu) for adult patients with unresectable or metastatic HER2-positive (IHC 3+) solid tumors after prior systemic treatment and without satisfactory alternative options. This represents the first tumor-agnostic HER2-directed ADC approval.

Trastuzumab Deruxtecan: mechanism and clinical evidence

T-DXd is an ADC combining trastuzumab with deruxtecan (DXd), a topoisomerase I inhibitor, through a cleavable tetrapeptide linker. Critical design advantages over T-DM1 include: (1) higher drug-to-antibody ratio (DAR ~8 vs. 3.5); (2) cleavable linker cleaved by lysosomal cathepsins and tumor microenvironmental proteases; (3) membrane-permeable DXd enabling diffusion into HER2-negative neighboring cells[46].

Recent mechanistic studies reveal T-DXd efficacy extends beyond classical HER2-mediated internalization. In HER2-low and HER2-negative breast cancers, T-DXd activity is largely independent of HER2 and instead relies on extracellular cathepsin L (CTSL) in the tumor microenvironment. CTSL efficiently cleaves the linker, releasing free deruxtecan in the extracellular space, where it can penetrate tumor cells irrespective of HER2 status. Invasive breast cancers abundantly express CTSL in both tumor and stromal compartments, providing a mechanistic explanation for T-DXd's activity even in tumors with minimal HER2 expression. Concurrently, T-DXd induces immunogenic cell death and activates myeloid cells via TLR4 and STING pathways, enhancing tumor antigen presentation and T-cell responses, suggesting that immune modulation may contribute to its therapeutic effect[46].

The DESTINY- Breast04[48] and DESTINY- Breast06[49] trials showed that patients with HER2- low expression (IHC 1+ or IHC 2+/ISH- negative), a population historically considered HER2- negative and ineligible for HER2- targeted therapies, derived substantial progression- free and overall survival benefit from T- DXd compared to conventional chemotherapy. This paradigm shift demonstrates that strict oncogene amplification and high- level protein overexpression are no longer absolute prerequisites for HER2- directed ADC efficacy. Instead, even modest, heterogeneous HER2 expression may be sufficient to confer therapeutic vulnerability, provided the drug platform is adequately designed to exploit low- density antigen expression through efficient internalization, high drug- to- antibody ratio, and membrane- permeable cytotoxic payloads.

Critical failure in Osteosarcoma: the PEPN1924 Trial

Enthusiasm for T-DXd in sarcomas was severely tempered by the PEPN1924 trial, a Pediatric Early Phase Clinical Trials Network study evaluating T-DXd in recurrent HER2-positive osteosarcoma. Eligibility required >10% tumor cells with cytoplasmic or membranous HER2

expression by immunohistochemistry; patients received T-DXd at standard dose (5.4 mg/kg every 3 weeks). Results were uniformly negative: Among 9 patients in the first stage, zero achieved objective response. Seven (78%) experienced progressive disease at first response evaluation after only 2 infusions, one withdrew consent, and one barely achieved stable disease at 24 weeks (the primary endpoint). The trial was stopped for futility.

Multiple mechanisms may explain this failure: (1) inclusion of cytoplasmic (non-membrane-accessible) staining, violating breast cancer-standard HER2 positivity criteria; (2) absence of ERBB2 amplification in enrolled patients, reflecting fundamentally different biology than amplification-driven carcinomas; (3) potential underdosing, though escalation would require careful phase I evaluation given T-DXd's interstitial lung disease risk; (4) intrinsic osteosarcoma resistance mechanisms independent of HER2 biology[50].

Contrasting preclinical success: DSRCT and implications for future patient selection

In contrast to osteosarcoma's failure, promising preclinical data emerged in desmoplastic small round cell tumor (DSRCT), an aggressive EWSR1::WT1 fusion-driven sarcoma with limited therapeutic options. In a recent preclinical study, trastuzumab deruxtecan (T-DXd) induced durable tumor regressions in DSRCT patient-derived xenografts, cell lines, and organoid models, with antitumor activity associated with rapid HER2-ADC internalization, p53-mediated apoptosis, growth arrest, and bystander payload effects that were proportional to HER2 expression levels [51]. In parallel, a monocentric clinical case series reported consistently high ERBB2 RNA expression in DSRCT and described marked, early, and clinically meaningful responses to T-DXd in three patients with metastatic refractory disease, all of whom achieved partial responses with ongoing treatment beyond 3 months[52]. These findings suggest that ERBB2/HER2-expressing sarcomas, particularly those with higher baseline expression and favorable tumor microenvironmental characteristics, may indeed benefit from HER2-targeted ADCs, warranting continued investigation.

CHAPTER 4 - RATIONALE AND AIMS OF THE PHD PROJECT

4.1 Hypothesis

Soft tissue and bone sarcomas are rare, heterogeneous tumors for which systemic therapy remains largely based on non- selective chemotherapy, with modest and short- lived benefits in the advanced setting. Molecular profiling has identified actionable drivers only in a minority of histologies, and most STS lack robust, druggable oncogenic addictions. In this context, there is a strong need for treatment strategies that exploit tumor- associated surface antigens rather than classical driver mutations.

HER2 is a prototypical example of such a target. While HER2 amplification and overexpression rarely reach "breast- like" levels in sarcomas, multiple lines of evidence indicate that HER2 can be expressed, often at low or intermediate density, in selected mesenchymal tumors and that ERBB2 alterations are enriched in specific STS subtypes. At the same time, next- generation antibody–drug conjugates such as trastuzumab deruxtecan (T- DXd) have demonstrated clinically meaningful activity not only in HER2- amplified carcinomas but also in HER2- low breast cancer, challenging the traditional notion that only high, homogeneous HER2 expression is therapeutically relevant.

This HER2- low paradigm, together with the recent tumor- agnostic approval of T- DXd for HER2- positive solid tumors, raises a critical and still unanswered question: can the levels and patterns of HER2 expression observed in soft tissue and bone sarcomas be functionally exploited by T- DXd, as already shown in epithelial tumors, or is HER2 in these malignancies merely a biologically incidental marker without therapeutic value?

The project at the core of this thesis was designed to address this gap. By quantitatively characterizing HER2 expression in a representative panel of sarcoma cell lines and testing the in vitro antitumor activity of T- DXd in these models, the work aims to define whether HER2 can serve as a practical and exploitable therapeutic target in selected sarcomas, and to identify which histologies are most likely to benefit from HER2- directed ADC strategies.

4.2 Specific objectives

- **Objective 1:** Quantitatively assess HER2 surface expression in a panel of soft tissue sarcoma and chordoma cell lines using flow cytometry, with HER2- high and HER2- low breast cancer cell lines as reference standards.

- **Objective 2:** Evaluate the in vitro antitumor efficacy of trastuzumab deruxtecan in HER2- expressing soft tissue sarcoma and chordoma cell lines, including chemoresistant models, and examine the relationship between HER2 expression levels and drug sensitivity.

CHAPTER 5 – MATERIAL AND METHODS

5.1 Cell lines

A panel of human sarcoma cell lines representative of distinct histological subtypes was selected to capture the biological heterogeneity of soft-tissue sarcomas. The panel included leiomyosarcoma (SK-LMS-1), liposarcoma (SW-872), gastrointestinal stromal tumor (GIST-T1), myxoid liposarcoma sensitive to trabectedin (402.91 WT) and its trabectedin-resistant counterpart (402.91 ET), as well as epithelioid sarcoma models (NEPS, ES-1 and ES-2). In addition, three chordoma cell lines derived from distinct anatomical sites and clinical presentations were included: CH-22, derived from a recurrent/metastatic sacral chordoma in a 56-year-old patient; U-CH17, derived from a sacral metastatic lesion in a 38-year-old patient; and U-CH14, derived from a clival primary tumor in a 79-year-old patient. Breast cancer cell lines with known HER2 status were used as biological controls, including SK-BR-3 (HER2-high) and MCF7 (HER2-low). Cell line provenance was as follows: 402.91 WT and 402.91 ET were kindly provided by Prof. Maurizio D'Incalci (Humanitas); chordoma cell lines were provided by the Chordoma Foundation; NEPS was kindly provided by Dr. Laurino (CROB, Rionero in Vulture); ES-1 and ES-2 were kindly provided by Dr. Sandro Pasquali (Istituto Tumori, Milan); GIST-T1 was kindly provided by Dr. Cesar Serrano (Vall d'Hebron University Hospital); and SK-LMS-1 and SW-872 were obtained from ATCC. Cells were maintained under standard humidified conditions at 37°C with 5% CO_2 and routinely passaged using standard detachment procedures according to line-specific requirements. Culture conditions followed supplier/provider recommendations; briefly, cells were grown in appropriate basal media (DMEM or RPMI-1640) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cell cultures were regularly monitored for morphology and growth kinetics, maintained within a limited passage range, and routinely tested to exclude mycoplasma contamination.

5.2 Flow Cytometry Analysis of HER2 Surface Expression

HER2 surface expression was quantified by flow cytometry and expressed as median fluorescence intensity (MFI). Cells were harvested during the logarithmic growth phase, washed with phosphate-buffered saline (PBS), and incubated with a fluorochrome-conjugated anti-HER2 monoclonal antibody (Alexa Fluor anti-human CD340/ErbB2/HER2 antibody, clone 24D2; BioLegend) directed against the extracellular domain. Following antibody incubation, cells were washed, resuspended in PBS containing 1% bovine serum albumin, and analyzed using a flow cytometer. After washing, cells were analyzed using a flow cytometer (Cytoflex, Beckman), measuring fluorescence intensity to determine surface expression levels. Data were analyzed using FlowJo software. Appropriate negative controls were included to define background fluorescence. For comparative purposes, HER2-high (SK-BR-3) and HER2-low (MCF7) breast cancer cell lines were analyzed in parallel and used as positive controls. All experiments were performed in at least three independent biological replicates.

ERBB2 transcript expression data for a subset of cell lines were obtained from RNA-seq analyses generated independently by collaborators at Centro Oncologico di Riferimento (CRO) in Aviano and were reported as protein-coding transcripts per million (pTPM). These data were used for exploratory correlation with T-DXd IC50 values.

5.3 Trastuzumab Deruxtecan exposure and drug assay

Trastuzumab deruxtecan (T- DXd) was purchased from Selleckchem and reconstituted according to the manufacturer's instructions to prepare a concentrated stock solution. Aliquots were protected from light and stored at –80 °C, then thawed immediately before use to avoid repeated freeze–thaw cycles. Serial dilutions were freshly prepared for each experiment to achieve the desired concentration ranges.

To assess the cytotoxicity of trastuzumab deruxtecan on sarcoma cell lines, we performed a cell viability assay. Briefly, cells were seeded in 96-well plates (10,000-12,000 cells/well) and allowed to adhere for 24 h. Cells were then treated with trastuzumab deruxtecan at the following concentrations: 0.1, 1, 5, 10, 20, 50, 100, and 200 μg/mL. After 72 h of treatment, cell viability was evaluated using an MTT assay (Thiazolyl Blue Tetrazolium Bromide).

The MTT assay is a colorimetric method based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) to purple formazan crystals by metabolically active cells. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes that reduce MTT to formazan. Formazan crystals were subsequently dissolved in a

solution of isopropanol and hydrochloric acid (HCl), and the resulting colored solution was quantified by measuring absorbance at 570/690 nm using a spectrophotometer (Tecan M200).

All drug- exposure experiments were performed in at least three independent biological replicates.

5.4 Statistical Analysis

Data were analyzed using Student's t-test. Graphical analyses were performed using GraphPad Prism software.

CHAPTER 6 - RESULTS: HER2 EXPRESSION IN SARCOMA CELL LINES

HER2 surface expression was quantified by flow cytometry across a panel of soft-tissue sarcoma, chordoma and epithelioid sarcoma cell lines, using breast cancer models as biological references. The HER2-amplified SK-BR-3 breast cancer control exhibited a marked rightward shift of fluorescence with a median fluorescence intensity (MFI) of 406000 and a HER2-positive fraction (mean) of 100 %, whereas the HER2-low MCF-7 breast cancer control displayed substantially lower staining (MFI 11679) with a HER2-positive fraction (mean) of 70.7%. In the conventional soft-tissue sarcoma panel, HER2 staining intensities were overall within the HER2-low range, with MFI values of 10346 in SK-LMS-1 leiomyosarcoma cells (HER2-positive mean 58.6%), 9995 in SW872 dedifferentiated liposarcoma cells (HER2-positive mean 45.9%), 10029 in GIST-T1 (HER2-positive mean 49.3%) and 5358 in 402.91 myxoid liposarcoma cells (HER2-positive mean 97.2%), indicating that inter-model variability was primarily driven by differences in the size of HER2-positive subpopulations rather than by uniformly high surface density (**Figure 5**).

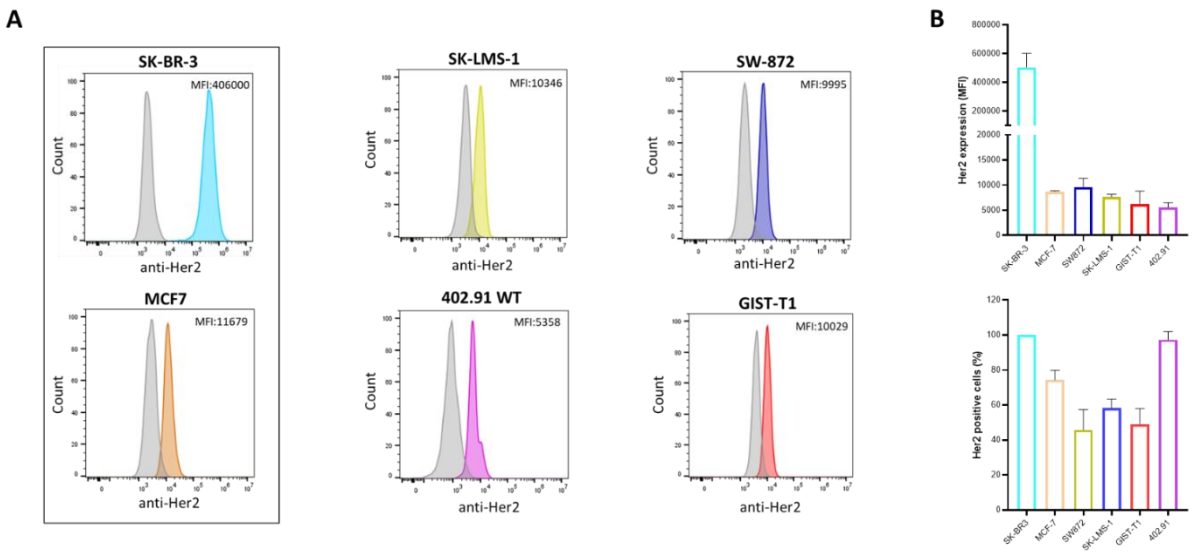

Figure 5. HER2 surface expression in conventional soft-tissue sarcoma cell lines and breast cancer controls. A. Representative images of HER2 surface staining in SK-LMS-1, SW872, GIST-T1 and 402.91 cell lines, with SK-BR-3 (HER2-amplified) and MCF-7 (HER2-low) as reference controls. **B**. For each model, the median fluorescence intensity (MFI) and the mean percentage of HER2-positive cells are reported. **Abbreviations: HER2**, human epidermal growth factor receptor 2; **MFI**, median fluorescence intensity.

HER2 expression was subsequently assessed in chordoma cell lines, confirming an overall low surface phenotype. MFI were 1565 in U-CH14, 1791 in U-CH17 and 2379 in CH22; within this subgroup, CH22 displayed the largest HER2-positive fraction (mean 41.3%) compared with U-CH14 (HER2-positive mean 12%) and U-CH17 (HER2-positive mean 26.3%) (**Figure 6**).

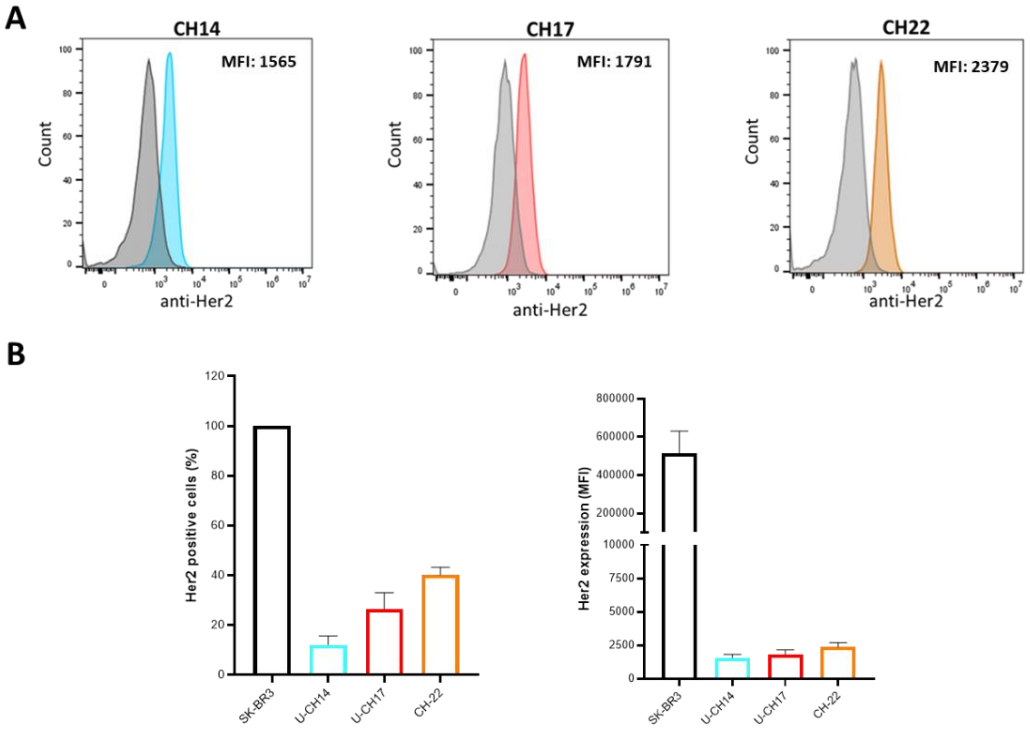

Figure 6. HER2 surface expression in chordoma cell lines. A. Representative images of HER2 surface staining in chordoma cell lines U-CH14, U-CH17 and CH22. **B.** MFI values and mean percentages of HER2-positive cells are reported for each line. **Abbreviations:** HER2, human epidermal growth factor receptor 2; MFI, median fluorescence intensity.

Epithelioid sarcoma models similarly exhibited very low HER2 surface staining. MFI values were 1549 for NEPS (HER2-positive mean 20.5%), 1900 for ES-2 (HER2-positive mean 25.1%) and 2862 for ES-1 (HER2-positive mean 62.3%), supporting the presence of heterogeneous HER2-positive subpopulations even within a uniformly low/very low intensity range (**Figure 7**).

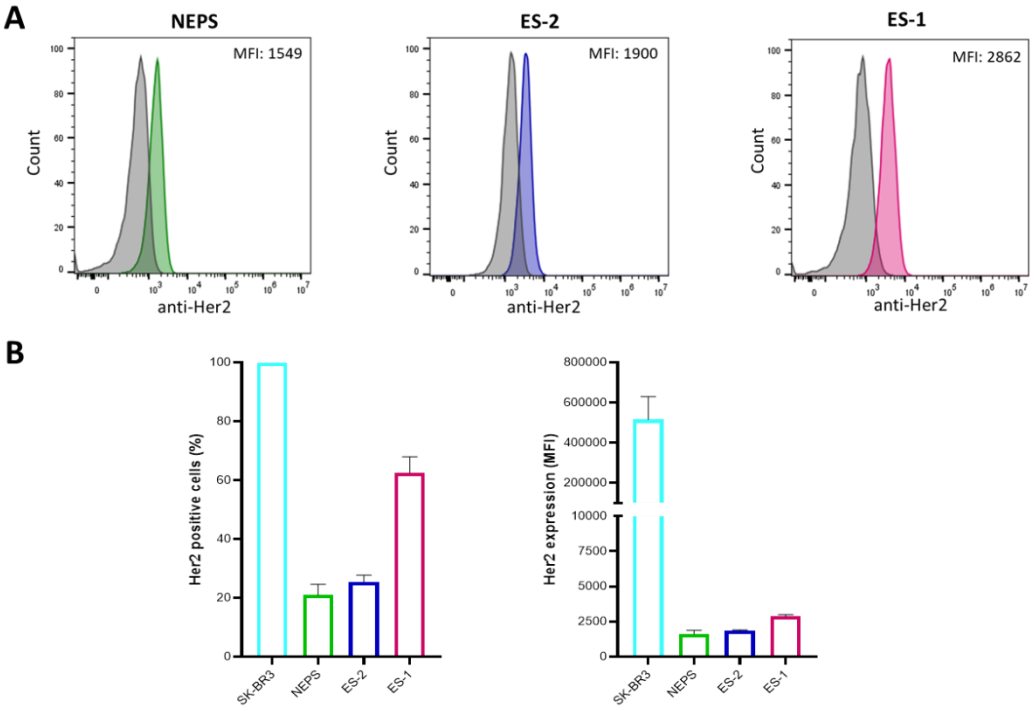

Figure 7. HER2 surface expression in epithelioid sarcoma cell lines. A. Representative images of HER2 surface staining in epithelioid sarcoma cell lines NEPS, ES-1 and ES-2. **B.** MFI values and mean percentages of HER2-positive cells are reported for each line. **Abbreviations: HER2**, human epidermal growth factor receptor 2; **MFI**, median fluorescence intensity.

CHAPTER 7 - RESULTS: ANTITUMOR ACTIVITY OF TRASTUZUMAB DERUXTECAN

The *in vitro* activity of trastuzumab deruxtecan (T-DXd) was evaluated across the same models by measuring cell viability after exposure to increasing drug concentrations, and IC50 values were derived from the dose–response profiles. Notably, neither HER2 median fluorescence intensity nor the fraction of HER2-positive cells appeared to correlate perfectly with T-DXd sensitivity across models, suggesting that additional determinants beyond surface HER2 abundance influence drug response. Consistent with its HER2-amplified status (MFI 406000, HER2-positive mean 100%), SK-BR-3 showed very high sensitivity with an IC50 of 0.081 μg/mL. The HER2-low breast cancer control MCF-7 showed an intermediate sensitivity profile, with an IC50 of 92.11 μg/mL, markedly higher than SK-BR-3 yet providing a clinically relevant pharmacologic comparator for the HER2-low setting. Among conventional soft-tissue sarcoma lines, the strongest activity was observed in 402.91 myxoid liposarcoma cells, a model characterized by low median intensity (MFI 5358) but a large HER2-positive compartment (mean 97.2%), with IC50 values of 29.37 μg/mL. By contrast, SK-LMS-1 leiomyosarcoma cells (MFI 10346, HER2-

positive mean 58.6%), SW872 dedifferentiated liposarcoma cells (MFI 9995, HER2-positive mean 45.9%) and GIST-T1 cells (MFI 10029, HER2-positive mean 49.3%) were less responsive, with higher IC50 values of 189.87 µg/mL, 215.98 µg/mL and 311.99 µg/mL, respectively (**Figure 8**).

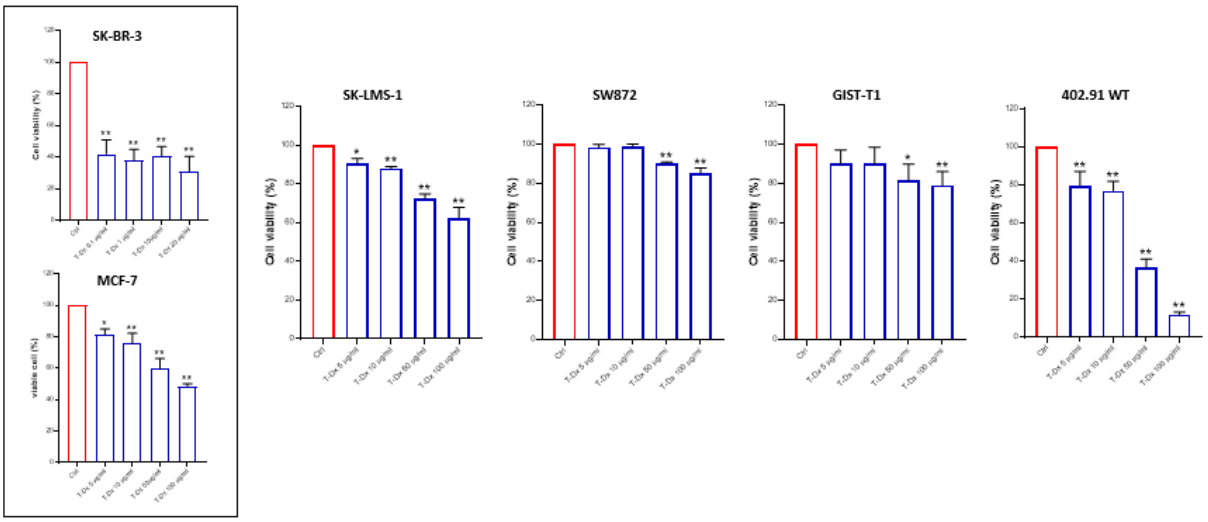

Figure 8. In vitro sensitivity to trastuzumab deruxtecan (T-DXd) in conventional soft-tissue sarcoma models. Cell viability following exposure to increasing concentrations of T-DXd in SK-BR-3 (HER2+ breast cancer cell line) and MCF 7 (HER2-low breast cancer cell line) and conventional soft-tissue sarcoma cell lines.

In chordoma, T-DXd elicited pronounced cytotoxicity despite the overall HER2-low phenotype. U-CH17 showed an IC50 of 55.44 µg/mL; CH22, which exhibited the highest HER2 signal among chordoma lines (median MFI 2379) and the largest HER2-positive compartment (mean 41.3%), was markedly more sensitive with an IC50 of 4.73 µg/mL (**Figure 9**).

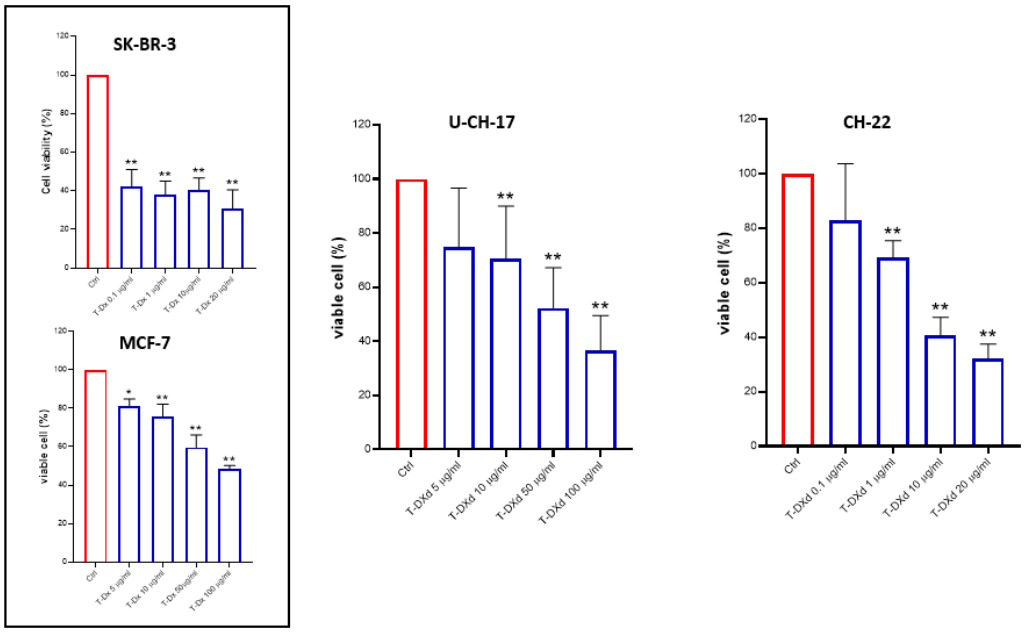

Figure 9. In vitro sensitivity to trastuzumab deruxtecan (T-DXd) in chordoma models. Cell viability following exposure to increasing concentrations of T-DXd in SK-BR-3 and MCF7 breast cancer cell lines and chordoma cell lines U-CH17 and CH22.

In epithelioid sarcoma, differential sensitivity to T-DXd was observed across models with low HER2 intensity but heterogeneous positivity. NEPS (median MFI 1549, HER2-positive mean 20.5%) was the most sensitive (IC50 41.20 µg/mL), followed by ES-1 (median MFI 2862, HER2-positive mean 62.3%; IC50 75.93 µg/mL), whereas ES-2 showed the lowest sensitivity (median MFI 1900, HER2-positive mean 25.1%; IC50 207.85 µg/mL), indicating that response ranking did not strictly parallel HER2 intensity or HER2-positive fraction (**Figure 10**).

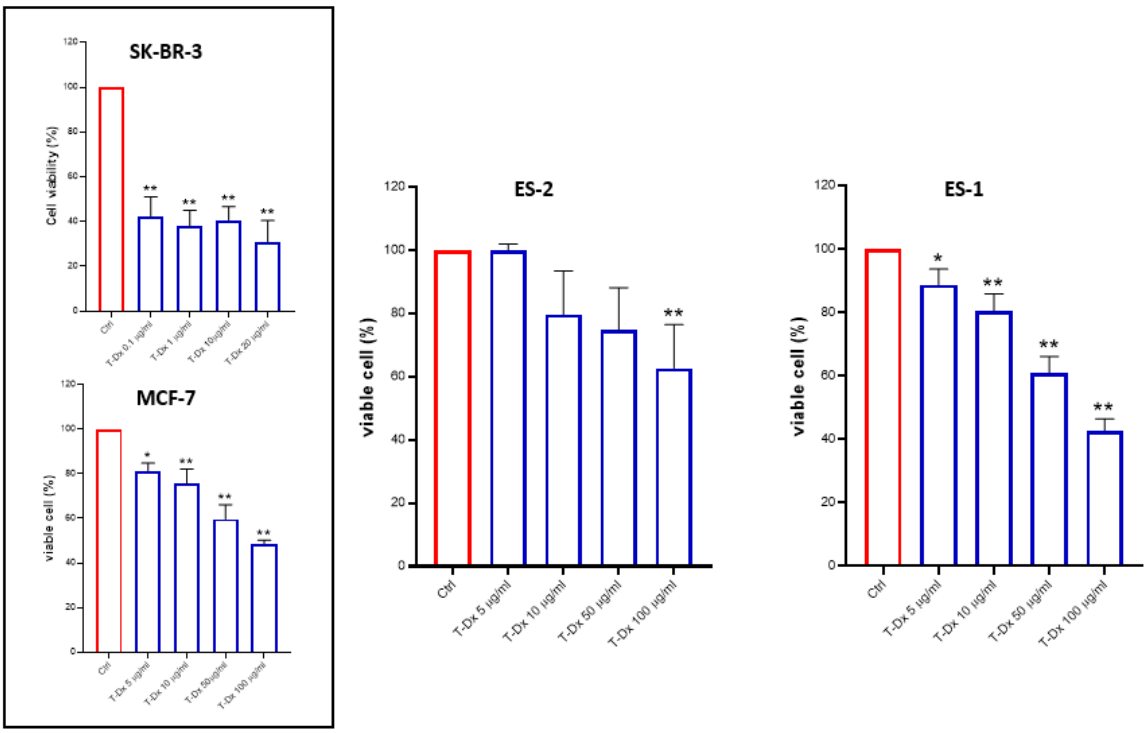

Figure 10. In vitro sensitivity to trastuzumab deruxtecan (T-DXd) in epithelioid sarcoma models. Cell viability following exposure to increasing concentrations of T-DXd in in SK-BR-3 and MCF7 breast cancer cell lines and epithelioid sarcoma cell lines (NEPS, ES-1 and ES-2**).**

Finally, HER2 expression was investigated in a paired myxoid liposarcoma model sensitive (402.91 WT) or resistant (402.91 ET) to trabectedin. Median fluorescence intensities were comparable between the two counterparts (5358 in WT and 5267 in ET), and the HER2-positive fraction (mean) remained high in both conditions (97.2% in WT and 92.0% in ET). Consistently, T-DXd activity was similar (IC50 29.37 µg/mL in WT and 26.71 µg/mL in ET), supporting the

notion that trabectedin resistance was not associated with a relevant reduction in HER2 availability in this model (**Figure 11**).

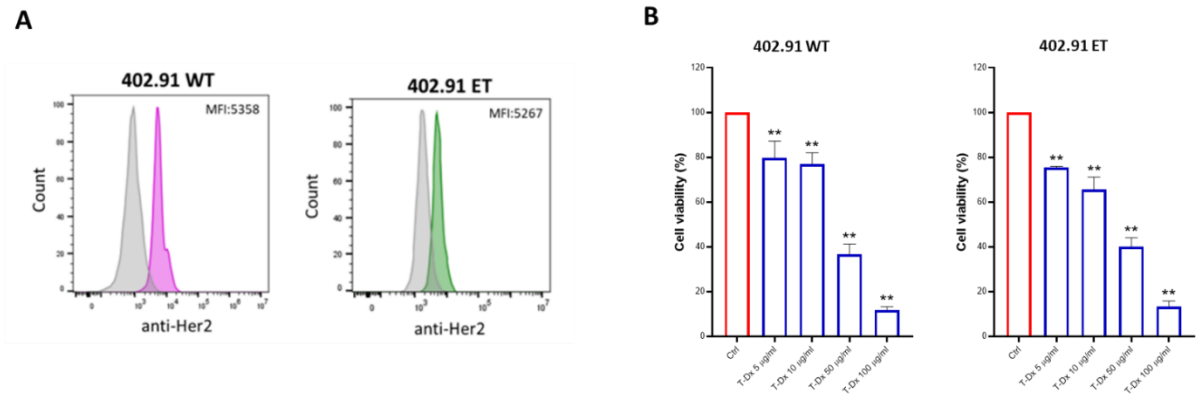

Figure 11. HER2 expression and T-DXd activity in a trabectedin resistance model (402.91 WT vs 402.91 ET). Comparison of HER2 surface expression (**A**) and T-DXd sensitivity (**B**) between trabectedin-sensitive (402.91 WT) and trabectedin-resistant (402.91 ET) myxoid liposarcoma counterparts.

Table 2 summarizes T-DXd IC_{50} values for all tested models and reports IC_{50} values normalized to MCF-7 (IC_{50} vs MCF-7).

Histotype	Cell line	HER2 MFI	HER2+ fraction (%)	T-DXd IC50 (µg/mL)	IC50 vs MCF-7
Breast cancer HER2+	SK-BR-3	406000	100	0.081	0.000879
Chordoma	CH22	2379	41.3	4.73	0.051352
Myxoid liposarcoma	402.91 ET	5267	92	26.71	0.289979
Myxoid liposarcoma	402.91 WT	5358	97.2	29.37	0.318858
Epithelioid sarcoma	NEPS	1549	20.5	41.2	0.447291
Chordoma	U-CH17	1791	26.3	55.44	0.601889
Epithelioid sarcoma	ES-1	2862	62.3	75.93	0.82434
Breast cancer HER2-low	MCF-7	11679	70.7	92.11	1
Leiomyosarcoma	SK-LMS-1	10346	58.6	189.87	2.06134
Epithelioid sarcoma	ES-2	1900	25.1	207.85	2.256541
DDLPS	SW872	9995	45.9	215.98	2.344805
GIST	GIST-T1	10029	49.3	311.99	3.387146

Table 2. Summary of HER2 surface antigenicity and T-DXd sensitivity across breast cancer controls and sarcoma cell lines. T-DXd half-maximal inhibitory concentration (IC_{50}, µg/mL) is shown for each model. IC_{50} vs MCF-7 represents the IC_{50} ratio relative to the HER2-low breast cancer comparator MCF-7 (ratio = 1). HER2 surface expression was quantified by flow cytometry and is reported as MFI (median fluorescence intensity) and HER2+ fraction (%) (percentage of HER2-positive cells). **Abbreviations**: **T-DXd**, trastuzumab deruxtecan; **IC_{50}**, half-maximal inhibitory concentration; **MFI**, median fluorescence intensity; **HER2**, human epidermal growth factor receptor 2; **STS**, soft tissue sarcoma; **DDLPS**, dedifferentiated liposarcoma; **GIST**, gastrointestinal stromal tumor.

In the subset of cell lines with available RNA-seq data, ERBB2 transcript levels (pTPM) showed a non-significant inverse correlation with T-DXd IC50 (Spearman's rho = -0.7, p = 0.233; n = 5). The highest ERBB2 expression was observed in 402 cells (pTPM 70), which also showed the lowest IC50 (29 μg/mL), whereas GIST-T1 cells displayed the lowest ERBB2 expression (pTPM 3) and the highest IC50 (312 μg/mL). Intermediate values were observed for ES-1 (36; 76 μg/mL), MCF-7 (57; 92 μg/mL), and ES-2 (48; 208 μg/mL), supporting a trend toward greater T-DXd sensitivity in models with higher ERBB2 expression.

CHAPTER 8 - DISCUSSION

The results of this study provide clear, quantitative evidence that low- to- moderate HER2 surface expression is consistently present across multiple sarcoma cell lines. Surface HER2 was detectable in all sarcoma cell lines by flow cytometry, yet remained quantitatively low relative to the HER2-amplified breast cancer control SK-BR-3 (MFI ~4×10^5), highlighting that ERBB2 in these sarcoma models behaves predominantly as a low-density surface antigen rather than a classic amplified oncogenic driver. Importantly, heterogeneity across sarcoma models was not captured by fluorescence intensity (MFI) alone, but also by the rate of the HER2-positive cell compartment. This was particularly evident in myxoid liposarcoma (402.91), where a low MFI coexisted with an almost uniformly HER2-positive population (~97%), and in epithelioid sarcoma ES-1, which displayed a large HER2-positive fraction (~62%) despite low signal intensity. In contrast, conventional STS lines (e.g., GIST-T1, SK-LMS-1, SW-872) clustered around a HER2-low intensity range with intermediate HER2-positive fractions, whereas chordoma models exhibited the lowest surface HER2 levels and smaller HER2-positive compartments overall, underscoring the broad, histotype-dependent diversity of HER2 antigenicity in sarcoma. Together, these data support a view of HER2 in sarcoma as a low-density yet heterogeneous surface antigen and provide a quantitative framework to interpret downstream ADC sensitivity beyond a simple "HER2-positive vs HER2-negative" dichotomy.

Notably, our results indicate that low-level surface HER2 can be compatible with T-DXd activity in vitro**,** including in some chemoresistant models. However, the magnitude of sensitivity varies substantially across histotypes and individual cell lines, revealing unexpected biological heterogeneity that challenges simple expression- response correlations and demands critical interpretation within broader preclinical and clinical contexts. Collectively these data do not support the paradigm in which HER2 expression uniformly translates into greater cytotoxicity,

suggesting that additional biological factors critically modulate T-DXd. Moreover, our findings challenge the long-held assumption that HER2, although historically detected in sarcomas, lacks therapeutic relevance, supporting a paradigm in which HER2 in sarcoma is better interpreted as a delivery address than as a driver alteration.

Among the results of this study there is the marked difference in T- DXd concentrations required to achieve cytotoxicity in sarcoma cell lines compared to HER2- amplified breast cancer controls. SK- BR- 3 cells, which express HER2 at extremely high levels (MFI=406,000), demonstrated profound sensitivity at sub- μg/mL concentrations, with significant cell death observed at 0.1 μg/mL and maximal cytotoxicity plateau at 10–20 μg/mL. The exceptional sensitivity of HER2- high models reflects the combination of extremely high receptor density, efficient receptor- mediated internalization, and robust intracellular payload accumulation following lysosomal processing. The inclusion of MCF-7 further refines this comparison by anchoring the analysis to a HER2-low epithelial model tested under the same experimental conditions. In our assay, MCF-7 exhibited substantially reduced sensitivity relative to SK-BR-3 (IC_{50} 92.11 vs 0.081 μg/mL), confirming that the HER2-low state can be associated with markedly attenuated in-vitro potency even in a tumor type where T-DXd clinical activity is well established. This observation supports the concept that standard 2D monoculture assays may under-represent in-vivo ADC efficacy in HER2-low contexts, in which microenvironmental factors may contribute to effective payload exposure.

Importantly, the pharmacodynamics of T-DXd in mesenchymal tumors may differ substantially from those observed in breast cancer. In addition to lower and more heterogeneous HER2 expression, sarcomas display a distinct stromal architecture, extracellular matrix composition, and protease milieu, all of which may influence linker cleavage, extracellular payload release, tissue penetration, and bystander effects. These factors are not adequately captured by conventional two-dimensional in vitro systems and may partly explain the divergence between nominal HER2 expression and drug sensitivity.

Recent mechanistic work offers a biologically plausible explanation for this apparent disconnect between attenuated in-vitro potency and established clinical benefit in HER2-low disease. Specifically, extracellular cleavage of the T-DXd linker by tumor-microenvironment proteases such as cathepsin L (CTSL) can promote local release of free deruxtecan (DXd), partially bypassing strict dependence on receptor-mediated internalization. In the absence of such microenvironmental components in standard monoculture assays, HER2-low models may therefore appear less sensitive in vitro than they are in vivo [46].

Similarly, most sarcoma models required tens to hundreds of μg/mL to achieve comparable viability reduction, and several IC50 values exceeded 200 μg/mL (e.g., SW872 ~216 μg/mL, ES-2 ~208 μg/mL, GIST-T1 ~312 μg/mL). This wide pharmacologic spread underscores that HER2 detectability alone does not guarantee clinically meaningful sensitivity under standard in vitro conditions.

Despite broadly detectable HER2, T-DXd cytotoxicity was highly heterogeneous, separating the models into distinct functional response patterns.

(i) Profound resistance even at very high concentrations. GIST-T1 displayed a strikingly limited maximal effect: even at 200 μg/mL, viability remained ~70%. This "low Emax" pattern suggests intrinsic resistance that is unlikely to be overcome simply by increasing exposure (and is therefore less consistent with meaningful single-agent activity at clinically tolerable dosing). Potential explanations include ineffective ADC internalization/processing, limited payload liberation, high efflux capacity, or reduced vulnerability to the topoisomerase-I payload.

(ii) Intermediate sensitivity with high concentration requirements. Leiomyosarcoma (SK-LMS-1) and dedifferentiated liposarcoma (SW872) showed dose-dependent effects but required very high concentrations to reach meaningful cytotoxicity (IC50 ~190 μg/mL and ~216 μg/mL, respectively). Within epithelioid sarcoma, sensitivity spanned a broad range despite uniformly low HER2 intensity: NEPS was the most sensitive (IC50 ~41 μg/mL), ES-1 showed intermediate sensitivity (IC50 ~76 μg/mL), whereas ES-2 was markedly less responsive (IC50 ~208 μg/mL). Notably, response ranking did not strictly parallel either HER2 intensity or the HER2-positive fraction (e.g., ES-1 had a larger HER2-positive compartment than NEPS yet was less sensitive), supporting the concept that HER2 alone is an incomplete predictor of T-DXd response. Similarly, also the U-CH17 chordoma line exhibited more substantial killing at higher concentrations (e.g., 50–100 μg/mL). These patterns imply that, under standard 2D monoculture conditions, T-DXd potency is borderline in many conventional STS/chordoma models.

(iii) "Breast-like" sensitivity in selected sarcoma models. Two models stood out as potentially actionable despite a HER2-low phenotype. First, the myxoid liposarcoma model 402.91 displayed consistent activity (IC50 ~29 μg/mL), and importantly this sensitivity was preserved in the trabectedin-resistant derivative (IC50 ~27 μg/mL), suggesting that acquired trabectedin resistance did not materially reduce HER2 accessibility or T-DXd vulnerability in this setting. Second, chordoma CH22 showed an IC50 of 4.73 μg/mL, over ten-

fold lower than U-CH17 (IC50 ~55 μg/mL), indicating that certain chordoma contexts may support "breast-like" sensitivity even at ultralow HER2 intensity.

A striking finding of this comparative analysis concerns the equivalent or even superior sensitivity of certain sarcoma models to T-DXd compared to the prototypical HER2-low breast cancer line MCF-7.

The discordant chordoma responses suggest that determinants beyond HER2 abundance are likely dominant. While CH22 also displayed a larger HER2-positive compartment, additional factors may include differences in ADC internalization and trafficking, lysosomal processing efficiency, linker-cleaving protease activity (e.g., cathepsins), and intrinsic vulnerability to topoisomerase-I inhibition (TOP1 abundance, replication stress and proliferation rate) as well as variability in DNA damage repair capacity and drug efflux. This CH22/U-CH17 pair therefore represents a useful platform for mechanistic follow-up aimed at identifying predictive biomarkers beyond HER2. Finally, if MCF-7-level T-DXd susceptibility translates into clinical benefit in breast cancer patients, then sarcoma models achieving equivalent or superior in vitro sensitivity (chordoma CH-22, epithelioid sarcoma NEPS, chordoma U-CH17, and myxoid liposarcoma) represent biologically plausible candidates for clinical response, directly rebutting the notion that sarcomas are intrinsically resistant to HER2-directed ADCs.

This observation has several implications. First, it challenges the conventional paradigm that sarcoma HER2 expression is "too low" to be therapeutically exploitable. In our models, mesenchymal tumors such as chordoma and epithelioid sarcoma, expressing HER2 at ultralow density (MFI 1,500–2,900), achieved T-DXd sensitivity equal to or better than MCF-7, a malignancy already demonstrated to benefit from T-DXd in randomized clinical trials. Second, the discordance between MCF-7 IC50 and sarcoma IC50 values, despite comparable absolute HER2 MFI in some cases, demonstrates that HER2 expression level alone is an incomplete predictor of ADC sensitivity.

Although not statistically significant, the observed inverse trend between ERBB2 transcript levels and T-DXd IC50 is biologically plausible and suggests that higher target expression may contribute to greater ADC sensitivity. However, given the very limited number of models and the exploratory nature of the RNA-seq dataset, this observation should be regarded as hypothesis-generating only.

While this pharmacokinetic integration provides a rational framework for clinical translation, several critical caveats must be acknowledged:

1. Two- dimensional monolayer culture does not recapitulate tumor microenvironment: our in vitro system lacks stromal fibroblasts, extracellular matrix, and immune cells that contribute to cathepsin L secretion and bystander payload distribution. Consequently, our IC_{50} values may underestimate in vivo efficacy if sarcoma tumor microenvironments are CTSL- rich, or overestimate efficacy if stromal barriers impair drug penetration.
2. Intratumoral PK variability: While average intratumoral T- DXd concentrations may reach 30–50 μg/mL, substantial inter- and intra- tumoral heterogeneity exists depending on vascularization, necrosis, and stromal composition. Poorly vascularized or fibrotic sarcomas may not achieve these concentrations.
3. Prolonged exposure effects not captured in 72- hour assays: Clinical T- DXd administration involves repeated cycles over weeks- to- months, enabling cumulative DNA damage, immunogenic cell death, and potential synergy with adaptive immune responses. Short- term viability assays may underestimate delayed or cumulative cytotoxic effects.
4. Resistance mechanisms not explored: This study did not assess mechanisms of intrinsic or acquired resistance (HER2 downregulation, ABCC1/MRP1 upregulation, enhanced DNA repair) that may limit clinical durability even in initially sensitive tumors.
5. HER2 was measured as surface MFI, which is quantitative and useful for rank-ordering, but does not directly map to clinical IHC scoring; integration with orthogonal assays (IHC/Western/quantitative proteomics) would strengthen translational interpretation.
6. The intrinsic variability of anti-HER2 antibodies used for diagnostic purposes, particularly in the setting of low-level expression, where differences in sensitivity and specificity may affect assay performance. While our assay showed good concordance in distinguishing positive and negative controls, most evaluated cell lines clustered within the low-to-very-low expression range; therefore, subtle gradations of HER2 expression may not have been captured with optimal accuracy.
7. Limited cell line panel. While the present study evaluated eight sarcoma cell lines across six histotypes, numerous clinically relevant sarcoma subtypes remain unrepresented. Notably absent are synovial sarcoma and MPNST cell lines, the two histotypes demonstrating the highest ERBB2 alteration frequencies in TCGA analyses). These histotypes represent priority targets for T-DXd investigation given their documented HER2 expression and poor prognosis.
8. Incomplete biomarker characterization. While HER2 surface expression was rigorously quantified by flow cytometry, this study did not assess complementary biomarkers that

may govern T-DXd response. Cathepsin B and L expression, critical mediators of linker cleavage and payload release, were not measured and may explain the differential T-DXd sensitivity observed across sarcoma histotypes. Similarly, topoisomerase I protein levels, DNA damage response pathway integrity (ATM, ATR, BRCA1/2 status), and drug efflux transporter expression (ABCB1, ABCC1) were not evaluated.

9. The lack of matched immunohistochemical assessment of HER2 in representative tissue samples from the corresponding sarcoma subtypes, which would have facilitated comparison between in vitro surface quantification and clinically used pathology-based classification.

Despite these limitations, the present findings provide compelling rationale for clinical investigation of trastuzumab deruxtecan in soft tissue sarcomas. The convergence of several lines of evidence (detectable HER2 expression across multiple histotypes, T-DXd cytotoxicity in vitro, activity in trabectedin-resistant models) establishes a biological foundation for translational research.

Patient selection strategies for early-phase clinical trials should prioritize histotypes with documented HER2 expression and high unmet need. Synovial sarcoma and malignant peripheral nerve sheath tumor, given their high ERBB2 alteration frequencies in TCGA (50% and 44%) and historical reports of HER2 protein expression, represent logical initial cohorts. Myxoid liposarcoma, particularly in trabectedin-refractory settings, constitutes additional high-priority populations given the in vitro efficacy demonstrated in this study. Chordoma and epithelioid sarcoma, as ultra-rare entities with limited therapeutic options, considering the efficacy results observed in our project, warrant inclusion in preclinical and clinical study designs despite lower HER2 expression levels.

Enrollment criteria should require HER2 expression by immunohistochemistry (IHC 1+, 2+, or 3+) with confirmation of membranous (not cytoplasmic) staining on archival or fresh tumor tissue. The PEPN1924 trial failure in osteosarcoma, which enrolled patients based on ≥10% cytoplasmic or membranous HER2 staining, underscores the importance of restricting eligibility to tumors with functionally accessible membrane-localized HER2. Central pathology reviews using standardized scoring criteria adapted from breast cancer guidelines would ensure consistency.

Biomarker-driven trial enrichment represents a critical opportunity. Beyond HER2 IHC, incorporation of cathepsin L immunohistochemistry or activity assays could identify patients most likely to benefit from cathepsin-mediated payload release. Similarly, assessment of topoisomerase I expression and DNA repair pathway integrity (BRCA1/2, ATM, PARP) could stratify patients

by predicted intrinsic sensitivity to deruxtecan. Prospective collection of these biomarkers, correlated with response outcomes, would refine predictive algorithms and enable adaptive trial designs enriching for biomarker-positive populations.

Based on these findings, a feasible early-phase clinical trial of HER2-directed ADCs in sarcomas should likely adopt a histology-enriched and biomarker-informed design. Candidate populations may include histotypes with reproducible HER2 surface expression or emerging clinical sensitivity signals, while eligibility should prioritize membranous HER2 expression assessed by standardized methods and, where feasible, integrate complementary biomarkers such as ERBB2 RNA expression, internalization-related features, and microenvironmental protease context. A window-of-opportunity or multi-cohort phase Ib/II design could represent a practical strategy to explore biological activity across selected mesenchymal tumors.

Conclusions

In conclusion, this doctoral project supports HER2 as a potentially exploitable surface antigen in selected sarcoma contexts**,** rather than a universal vulnerability across mesenchymal tumors. By quantitatively defining HER2 antigenicity (both intensity and HER2-positive fraction) and demonstrating highly heterogeneous T-DXd sensitivity, this work identifies distinct "signal" models, most notably chordoma CH22 and myxoid liposarcoma 402.91 (including a trabectedin-resistant derivative), in which T-DXd activity emerges at substantially lower concentrations than in many conventional STS lines. At the same time, the presence of intrinsically refractory models with IC50 values exceeding 200 μg/mL highlights the need for predictive biomarkers beyond HER2 alone, potentially encompassing determinants of payload activation/processing and intrinsic susceptibility to topoisomerase-I inhibition. Overall, these findings provide a rationale for biomarker-enriched translational studies and early-phase clinical trial designs prioritizing histotypes and biological contexts where clinically meaningful exposure-response relationships are most plausible.

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