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New therapeutics for soft tissue sarcomas

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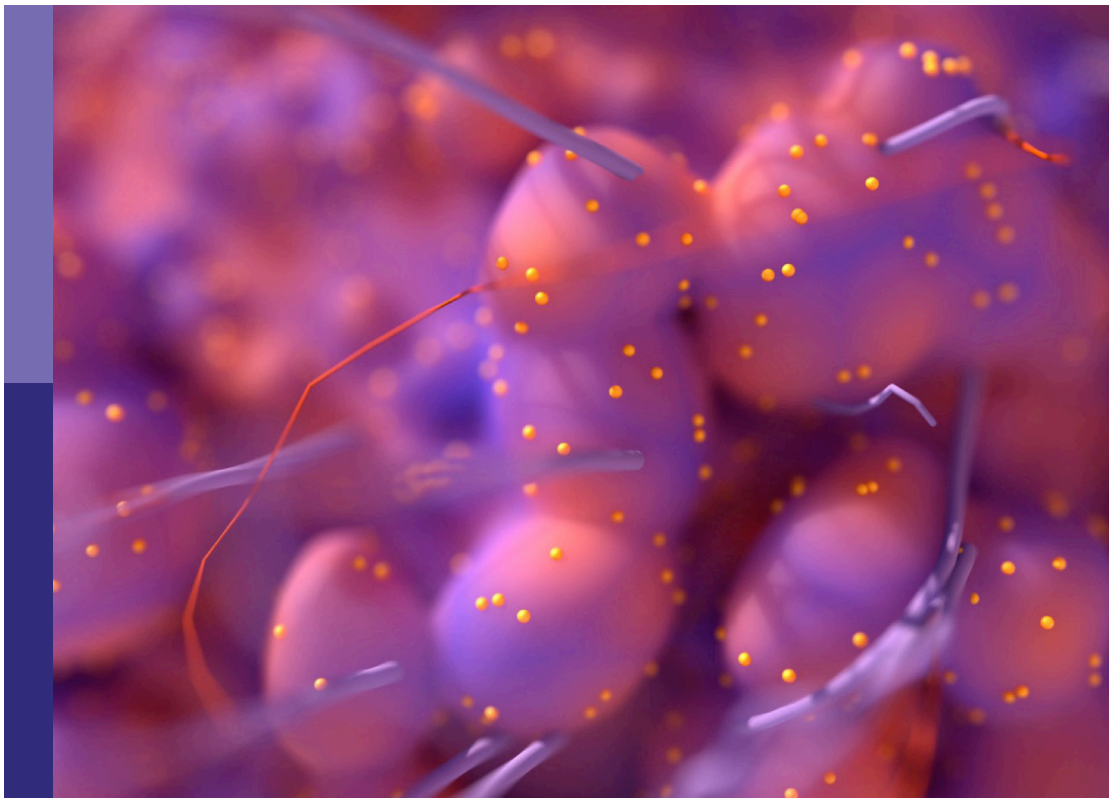

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New therapeutics for soft tissue sarcomas

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Editorial: New therapeutics for soft tissue sarcomas

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sarcoma, combination (combined) therapy, chemotherapy, immunotherapy, personalized medicine

Editorial on the Research Topic
New therapeutics for soft tissue sarcomas

Soft tissue sarcomas (STS) represent less than 1% of all tumors and are thought to be derived from mesenchymal progenitor cells. This heterogeneous class of tumors include over 100 histological subtypes with varying clinical presentation and genetics. Their heterogeneity reflects the ability of mesenchymal precursors to develop into an array of tissue types including muscle, bone, cartilage, and fat.

There are no routine standard screening tests for sarcoma, but once a tumor is detected the prognosis is based on the disease stage, histopathology, size, and genetics. Standard of care includes surgery, radiation and chemotherapy. A percentage of sarcomas have actionable driver mutations, but most do not. STS are typically "cold", with a low tumor burden and are not likely to respond to immunotherapy. Despite improvements in therapy, the 5-year survival rate remains low, reported as 58% (Cancer Net, ASCO) due in part to higher frequencies of advanced metastatic disease.

This Research Topic includes nine multidisciplinary manuscripts. One overall theme is that targeted therapies can be very effective for specific STS with specific actionable mutations, but only a limited percentage of sarcoma patients have these mutations. Even patients that respond well to targeted therapy often relapse, and then require subsequent non-targeted therapy. Therefore, additional therapies that are both for targeted sarcomas and for sarcomas lacking obvious actionable genetics are required that can prevent relapse/prolong PFS.

Fuchs et al., reviewed FDA approved drugs for STS. Pazopanib is FDA approved for non-adipocyte STS based on clinical prolongation of the PFS from 1.6 months to 4.6 months. The OS increase did not reach statistical significance (10.7 vs 12.5 months). Other drugs reviewed were approved for more targeted populations and have longer response rates. (1) Pexidartinib (CSF1R, Ckit inhibitor) for tenosynovial giant-cell tumors (locally invasive, frequently due to CSF1 overexpression), 39% overall response rate (2) imatinib for dermatofibrosarcoma protuberans (locally invasive with COL1A1/PDGFB fusion protein), 5% CR, 55% PR, (3) crizotinib for ALK positive inflammatory myofibroblastic tumors (locally invasive), (4) tazemetostat (EZH2 inhibitor) for epithelioid sarcoma (can be

metastatic; characterized by loss of INI1 subunit of the SWI/SNF chromatin remodeling complex that opposes the enzymatic function of EZH2), (5) nab-sirolimus for perivascular epithelioid cell tumor (PEComa, often seen with tuberous sclerosis), (6) tropomyosin receptor kinase inhibitors for TRK fusion positive cancers. Also reviewed were targeted kinase inhibitors that do not have FDA approval for STS but are in clinical testing.

Maleddu et al. reviewed only locally aggressive mesenchymal tumors, particularly Desmoid fibromatosis, giant cell tumor of bone, and tenosynovial giant cell tumor. These tumors have limited ability to metastasize and therefore carry a better prognosis than metastatic tumors. As noted by Fuchs et al. as well, treatment for these includes targeted chemotherapy approaches that can be effective. New therapies and paradigms have focused on utilizing less toxic regimens and identifying patients that do not require more toxic regimens.

Fuchs et al. reviewed new therapeutics for synovial sarcoma (SYN). SYN is defined by the translocation of t(X:18) (p11.2;q11.2) forming an oncogenic fusion protein, with about 1000 cases per year in the US (1). Although SYN is more responsive to chemotherapy, the overall survival is worse than that of most STS. He reviewed clinical trials using adoptive cell transfer, where autologous T cells are transfected with engineered T-cell receptors that bind antigens that are expressed predominately in SYN including NY-ESO-1, PRAME, and MAGE-A4. Included is a description of the challenges with the approach.

Seong and D'Angelo reviewed immune approaches for STS. STS generally have low levels of infiltrating lymphocytes and may have higher levels of immunosuppressive M2 macrophages.

Lacuna et al. reviewed new therapeutics for leiomyosarcoma (LMS). First-line therapies are anthracycline- or gemcitabine-based regimens, resulting in a median PFS time of about 5 months and overall survival time between 14-16 months. LMS is not typically associated with specific mutations, but with complex karyotypes. Highlighted in the review are new therapies based upon: (1) DNA repair deficiencies because LMS is often associated with defects in DNA-repair. This includes a Ph2/3 trial assessing temozolomide and Olaparib in uterine LMS. (2) Kinase inhibitors, including cabozantinib and anlotinib. (3) Metabolic vulnerabilities due to the frequent loss of argininosuccinate synthase 1. (4) New chemotherapeutic combinations including a Phase 3 trial of unesbulin with dazacarbazine. Unesbulin is an orally bioavailable inhibitor of tubulin polymerization. Possibly, the combination of unesbulin (causes G2M arrest) and dazacarbazine (an alkylating agent) causes replicative stress, particularly in tumors such as LMS that are deficient in DNA-repair.

Three papers discuss new therapies tested in preclinical studies or in a case report. First, Bernardo et al. demonstrates that both photon and proton irradiation are equally effective in a sarcoma mouse model. In clinical trials, first line therapy is the combination of radiotherapy with doxorubicin, although the rates of recurrence remain high. Clinical trials are not conducted to optimize radiation/chemotherapy regimens or to compare types of radiation, increasing their value in preclinical studies. Second, Marritt et al. present preclinical data demonstrating efficacy of STING agonists in models of undifferentiated polymorphic sarcoma UPS. Third, Li et al. describe a case report of a favorable response of an ALK-fusion positive protein to the ALK TKI ensartinib. Epithelioid inflammatory myofibroblastic sarcoma (EIMS) is often associated with ALK fusion proteins.

Finally, because osteosarcomas show genetic similarities to soft tissue sarcomas, we have included a paper describing a prognostic score predicting responsiveness to high dose methotrexate (Ganguly et al.). Tumor size, baseline metastases and SAP were prognostic factors to predict survival, but social factors were not. Similar parameters are likely to be relevant to STS tumors.

In summary, new therapies are being identified and developed for sarcomas. Some of these are targeted therapies leveraging the gains made in personalized medicine. Others are focused on novel chemotherapeutic combinations leveraging the complex karyotype of sarcoma on the background of DNA repair deficiencies. Exciting new immuno-oncology approaches are in preclinical and clinical stages. Some of these approaches may provide new therapies to overcome resistance and provide more possible treatments to mix and match in varying sequence so as to provide measurable benefit to STS patients.

Author contributions

MW: Conceptualization, Writing – original draft, Writing – review & editing. MR: Writing – original draft, Writing – review & editing. MI: Conceptualization, Writing – review & editing. BT: Conceptualization, Writing – review & editing.

Conflict of interest

MW and MR are employees of PTC Therapeutics. In connection with such employment, they receive salary, benefits and stock-based compensation. MI is an employee of Regeneron. In connection with such employment, he receives salary, benefits, and stock based compensation. Further declarations are outlined below: Consulting or Advisory Role: Daiichi Sankyo, Xencor, Apexigen, Epizyme, Caris Life Sciences Research Funding: Apexigen Inst, Mirati Therapeutics Inst, PTC Therapeutics Inst, Intensity Inst, Boehringer Ingelheim Inst, Bioatla Inst, Merck Inst, Astellas Pharma Inst, AstraZeneca Inst For BT, his declarations are outlined below: Consulting: Cytokinetics Inc Consulting/Advisor 2020 exp. 7/23 Bayer Consulting/Advisor 2021 Deciphera Pharm, Extended the consulting relationship 9/2022. Daiichi Sankyo Inc. Consulting 2021, 2022 EcoR1 Consulting 9/2022 Advenchen Consulting Putnam Consulting 2023 Salarius Pharm, Inc., Consulting 2023 Boxer Capital LLC Consulting 2023 Acuta Capital, LLC Consulting 12/2022 5 years Participation on a Data Safety Monitoring Board or Advisory Board Apexigen Inc Advisory Board Meeting 2022 Daiichi Sankyo Advisory Board Meeting 2021, CTOS 2022 Bayer US Medical Affairs Oncology Virtual Advisory Board Meeting 11/30/21 2022 PTC Therapuetics Advisory Board Meeting 2022 Aadi Biosciences CTOS advisory board, PRECISION 1: Exploring Best Practices for a Tumor Agnostic Study in an Organ-specific World 11/22 Boehringer Ingelheim Advisory Board

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Intratumoral STING activation causes durable immunogenic tumor eradication in the KP soft tissue sarcoma model

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Introduction: Soft tissue sarcomas (STS) are highly metastatic, connective-tissue lineage solid cancers. Immunologically, sarcomas are frequently characterized by a paucity of tumor infiltrating lymphocytes and an immune suppressive microenvironment. Activation of the STING pathway can induce potent immune-driven anti-tumor responses within immunogenic solid tumors; however, this strategy has not been evaluated in immunologically cold sarcomas. Herein, we assessed the therapeutic response of intratumoral STING activation in an immunologically cold murine model of undifferentiated pleomorphic sarcoma (UPS).

Materials and Results: A single intratumoral injection of the murine STING agonist, DMXAA resulted in durable cure in up to 60% of UPS-bearing mice. In mice with synchronous lung metastases, STING activation within hindlimb tumors resulted in 50% cure in both anatomic sites. Surviving mice all rejected UPS re-challenge in the hindlimb and lung. Therapeutic efficacy of STING was inhibited by lymphocyte deficiency but unaffected by macrophage deficiency. Immune phenotyping demonstrated enrichment of lymphocytic responses in tumors at multiple timepoints following treatment. Immune checkpoint blockade enhanced survival following STING activation.

Discussion: These data suggest intratumoral activation of the STING pathway elicits local and systemic anti-tumor immune responses in a lymphocyte poor sarcoma model and deserves further evaluation as an adjunctive local and systemic treatment for sarcomas.

KEYWORDS

cancer immunotherapy, cGAS/STING, undifferentiated pleomorphic sarcoma, KP sarcoma model, soft tissue sarcoma

Introduction

Soft tissue sarcomas (STS) are rare malignancies derived from mesenchymal lineage tissues such as muscle, adipose, fibrous tissue, vessels, and skin (1). Sarcomas are rare, representing <1% of all cancer diagnoses, yet disproportionately account for 15-20% of solid cancers in children, adolescents, and young adults (2–4). There are over 50 unique histologic subtypes of STS (1), with undifferentiated pleomorphic sarcoma (UPS) being the most common subtype in adults (4). High-grade soft tissue sarcomas are considered a high-fatality disease characterized by frequent metastases, resistance to systemic therapies, and a five-year survival rate of under 60% (1, 5). Unresectable metastatic disease is rapidly fatal (1, 6–8) and there is a pressing need for new systemic therapies for STS patients (9).

Immunotherapies are revolutionizing cancer care (10–13), yet unfortunately, sarcoma remains recalcitrant to multiple clinically approved immune-based therapies (14–18). Relative to other solid cancers, most sarcomas are deficient in tumor infiltrating lymphocytes (TILs) (19–21), which, like other solid cancers, predicts poor therapeutic responses to immune checkpoint inhibition (ICI) (22). The immunosuppressive landscape of STS is multifactorial and can be attributed to a combination of low tumor mutational burden, dense infiltration of immune suppressive macrophages ("M2-like" macrophages), and the expression of immune suppressive connective tissue cytokines and growth factors within mesenchymal-derived sarcomas (23–25).

The stimulator of interferon genes (STING) receptor is a highly conserved intracellular protein involved in the dsDNA sensing apparatus of eukaryotic cells and is responsible for Type I IFN and cytokine production in response to cytosolic DNA derived from pathogens and corrupt host cells (26, 27). The STING pathway provides a critical link between the innate and adaptive compartments of the immune system and is a vital component of cancer immunity (19, 21, 28). When STING is activated, the potent liberation of Type I IFNs and other inflammatory mediators results in tumor necrosis (19, 28), activation of antigen presenting cells (APCs) (25, 28), enhanced cross-priming of $CD8^+$ lymphocytes and recruitment of anti-tumor lymphocytes into the tumor immune microenvironment (TIME) (19, 21, 28). In pre-clinical models of classically inflamed solid tumors, intratumoral (i.t.) small molecule STING agonists can induce dramatic local tumor regression and systemic immunity against distant disease and this strategy has now entered early phase clinical trials.

STING immunotherapy has not been evaluated in immunogenically cold models of STS. As poorly inflamed sarcomas are recalcitrant to immune-based therapies such as immune checkpoint inhibitors (14, 17–19, 21), we hypothesized that i.t. STING therapy would be an effective strategy to dismantle the immune suppressive sarcoma microenvironment and sensitize murine STSs to ICI. Herein, we evaluated the therapeutic anti-tumor effects of STING activation in a lymphocyte poor murine model of UPS that is resistant to ICI (29–31). We demonstrate that that a single i.t. dose of a small molecule STING agonist resulted in rapid immune-mediated tumor clearance locally and systemically and therapeutic synergy with immune checkpoint blockade.

Materials and methods

Mice

All *in vivo* murine studies were performed animal use protocols approved by the University of Calgary Health Sciences Animal Care Committee (#AC19-0072). Mice were housed in a biohazard level 2 containment facility in individual cages (Techniplast) equipped with HEPA filters and filtered air. The mouse housing room was maintained at 22 +/- 1°C, 30-35% humidity, and was on a 12-hour light/dark cycle. The mice were allowed standard food and water *ad libitum*. All *in vivo* murine experiments were performed in 6–8-week-old male and female mice. All mice were purchased from Jackson Laboratories and then bred in house. Rag2 KO mice (B6(Cg)-$Rag2^{tm1.1Cgn}$/J Rag2 knockout mice; stock #008449) are deficient in mature T-cells and B-cells (32). CCR2 KO mice (B6.129S4-$Ccr2^{tm1Ifc}$/J CCR2 knockout mice; stock #027619) show a monocyte recruitment deficiency to sites of inflammation and were used to test tumor macrophage deficiency (33).

Tumor model

The development of the syngeneic KP UPS cell line used in these experiments is described and characterized by Hildebrand et al. (2021) (29), and also previously by DuPage et al. (2012) (31) and Kirsch et al. (2007) (30). Briefly, spontaneous UPS tumors were generated in conditional $Trp53^{fl/fl}$ and $Kras^{G12D/+}$ mice *via* lenti-Cre (University of Iowa Viral Vector Core; FIVCMVCre VSVG) mediated *Trp53* deficiency and activation of the $Kras^{G12D}$ oncogene subperiosteally in the hindlimb of female C57Bl/6 mice which results in establishment of primary UPS tumors exclusively in the proximal tibia of the hindlimb. Following a latency of 8-10 weeks, hindlimb tumors were harvested and cultured *in vitro* for 6-8 weeks for cell line development. Only cell line derived tumors were used as the model for this project. Cultured UPS tumor cells were engineered to express mCherry and firefly luciferase *via*

transduction with pLV430G oFL T2A mCherry vector. This cell line is referred to as "TAO1+". Aliquots of UPS cell not transduced with mCherry and luciferase are referred to as "TAO1-". All UPS tumors evaluated in *in vivo* experiments reported in this study utilized engrafted TAO1+ UPS tumors in which UPS cells were resuspended in serum free RPMI-1640 media and injected intramuscularly into the right hindlimb. The quantity of UPS cells injected were as follows: 100,000 for primary injection, 10,000 for contralateral limb injection, and 100,000 for tail vein injection.

Tumor volume assessment and bioluminescent imaging

Tumors were monitored by caliper measurements and bioluminescent imaging (BLI). For BLI, mice were injected with D-luciferin (Goldbio Technology; cat. #LUCK-1G) intraperitoneally and imaged using a Xenogen IVIS Lumina system (Caliper Life Sciences, Hopkinton, MA, USA) ten minutes following injection. Living Image Software (PerkinElmer) to collect and analyze the BLI images. The image exposure was set to "Auto."

Caliper measurements were used to measure tumor length, width, and depth. Length is defined as proximal to distal, width is defined as lateral to medial, and depth is defined as anterior to posterior measurements. Tumor volumes were calculated with the formula (L+X)*L*X*0.2618, where L is the length of the tumor and X is (width of tumor + depth of tumor)/2 (29, 34). The humane endpoint for any mouse experiment was defined as a leg tumor exceeding 15 mm in the length, width, or depth dimensions. For the tail vein injection experiments and any mice with lung tumors, the humane endpoint was defined as any rapid deterioration of overall health including rapid weight loss, loss of grooming, hunched posture, and lethargic behavior. Experimental endpoint for any murine long-term survival experiment was defined as three months after primary cell line injection, one month after contralateral limb re-challenge, and two months after tail vein re-challenge. All mice alive beyond these experimental timelines are regarded as "survivors." We have not observed any evidence of UPS relapse after these experimental endpoints.

5'6-dimethylxanthenone-4-acetic acid experiments

In this study, DMXAA was used to investigate STING immunotherapy in murine UPS tumors. In all experimental groups, 100,000 UPS cells were injected into the right hindlimb muscle of C57Bl/6 mice. Intra-tumoral (i.t.) injection(s) of DMXAA (Sigma; cat. #D5817-25MG) or sodium bicarbonate (Gibco; cat. #25080094) were administered when UPS tumors reached ~100 mm^3 (7 days after cell line engraftment). The experimental groups were: (i) one DMXAA (18 mg/kg) injection (n=10), (ii) one DMXAA (25 mg/kg) injection (n=14), (iii) two DMXAA (18 mg/kg) injections (n=10), (iv) three DMXAA (18 mg/kg) injections (n=10), and (v) sodium bicarbonate vehicle controls (n=9). For (i), (ii), and (v) the treatment was delivered 7 days post UPS injection. For experiment (iii) DMXAA was administered 7- and 14-days post UPS injection. For experiment (iv) DMXAA was administered 7-, 11-, and 14-days post UPS injection. An additional cohort was utilized in which 100,000 UPS cells were injected into the tail vein for lung engraftment on day 0, followed by concurrent leg tumor engraftment of 100,000 UPS cells on day 7. On day 14, 18 mg/kg of DMXAA was administered i.t. in the hindlimb. Single and double DMXAA doses were chosen based on previous studies (24, 28, 35). The triple DMXAA dosing was modified from this same study. For the Rag2 and CCR2 KO mice experiments, 100,000 UPS cells were injected into the right hindlimb muscle. DMXAA (18 mg/kg) or sodium bicarbonate vehicle control were injected i.t. when tumors reached ~100 mm^3 (7 days after cell line engraftment).

In vivo re-challenge experiments

Mice from the cohort that were engrafted with 100,000 UPS cells in the right hindlimb, subsequently received an i.t. dose of DMXAA (18 mg/kg) and survived were re-challenged with UPS cells. Survival was characterized as mice that are tumor free with no evidence of tumor after three months. For the re-challenge experiments in the primary site, 10,000 UPS cells were injected into the muscle of the contralateral hind limb of "survivors" and naïve C57Bl/6 mice. For the tail vein re-challenge experiments, 100,000 UPS cells administered through a tail vein injection in "survivors" and naïve C57Bl/6 mice. Tail vein injections of murine UPS cells into C57Bl/6 mice had been previously determined by our laboratory to result in UPS tumors exclusively in the lung within 3-4 weeks using this model. Weekly BLI and overall mouse health were used to assess tumor growth.

Immune checkpoint inhibitor therapy

Mice bearing syngeneic UPS hindlimb tumors were treated with a mouse anti-CTLA4 monoclonal antibody (BioXcell, CD152, clone 9D9, 250 μg) or a mouse anti-PD1 monoclonal antibody (BioXcell, CD279, clone RMP1-14, 250 μg) intraperitoneally, on days 7, 10, and 13 following UPS injection. For anti-PD-1 + anti-CTLA4 dual therapy, UPS-bearing mice were treated with mouse anti-CTLA4 (BioXcell, CD152, clone 9D9, 250 μg) and mouse anti-PD1 (BioXcell,

CD279, clone RMP1-14, 250 µg) intraperitoneally, on days 9, 11, 15, 18, 22, 25, 29, and 32 following UPS injection. For DMXAA + anti-PD-1 + anti-CTLA4 combination therapy, UPS-bearing mice were treated with i.t. DMXAA (18 mg/kg) on day 7 and mouse anti-CTLA4 (BioXcell, CD152, clone 9D9, 250 µg) and mouse anti-PD1 (BioXcell, CD279, clone RMP1-14, 250 µg) intraperitoneally, on days 9, 11, 15, 18, 22, 25, 29, and 32 following UPS injection.

Flow cytometry

100,000 UPS cells were engrafted into the hind limb muscle of C57Bl/6 mice and 7 days later when the tumors reached a tumor volume of ~100 mm^3, i.t. injections of DMXAA (18 mg/kg) or sodium bicarbonate were given. Tumors were processed for flow cytometry 3- and 7-days post DMXAA or sodium bicarbonate treatment. UPS tumors were excised and homogenized using a gentleMACS Dissociator (Miltenyi). Tumors were digested with RPMI-1640 media (Gibco; cat. #22400089) containing 0.5 mg/mL DNAse I (Roche Diagnostics; cat. #10104159001), 20 mg/mL Collagenase II (Gibco; cat. #17101-015), and 0.5 mL/10 mL fetal bovine serum (Gibco; cat. #12483-020). Tumors were then strained with a 70 µm strainer (Falcon™; cat. #08-771-2), treated with RBC lysis buffer (Biolegend; cat. #420301), and washed with 40% Percoll™ (cat. #17-0891-02).

Single cell suspensions were stained with LIVE/DEAD Zombie Aqua (cat. #423102) before antibody staining for 15-30 minutes. Antibody staining was completed using the following fluorophore-conjugated antibodies: CD3ϵ (cat. #155609), CD4 (cat. #100512), CD8α (cat. #100733), CD45 (cat. #103154), CD11b (cat. #101207), Ly6C (cat. #128005), and Ly6G (cat. #127615). Data was acquired using a FACSCanto II (BD Biosciences) with FACSDiva software (BD Biosciences). The data was analyzed with FlowJo (TreeStar). T-cells were defined as CD3ϵ+/CD4+ (CD4 T-cells) and CD3ϵ+/CD8+ (CD8 T-cells). Monocytes were defined as CD45+/CD11b+/Ly6C+/Ly6G-, neutrophils as CD45+/CD11b+/Ly6C+/Ly6G+ and macrophages as CD45+/CD11b+/Ly6C-/Ly6G-. Controls included a dead cell sample, achieved by heating the tumor cells to 80°C for 15 minutes, unstained tumor cells, and single colour controls. Single colour controls were made using compensation beads (Invitrogen) (cat. #501129040).

mRNA quantification and analysis

NanoString® technology was used to compare the mRNA expression levels of ~750 genes in the following four treatment groups: control UPS tumors (n=4), UPS tumors 24 hours post DMXAA treatment (18 mg/kg; n=4), and UPS tumors 72 hours post DMXAA treatment (18 mg/kg; n=7). Total RNA was extracted from TAO1+ UPS tumors using standard protocols. 100 µg of unamplified total RNA input was used for codeset hybridization using the mouse-specific nCounter® PanCancer Immune Profiling panel (NanoString® Technologies, Seattle, WA) (36). Codeset/RNA complexes were immobilized on nCounter® cartridges for data collection. nSolver Analysis Software 4.0 and Advanced Analysis were used for analysis and figure generation.

Histopathology

UPS tumors were fixed in 10% neutral buffered formalin (Research Products International Corp) for 24 hours and embedded into paraffin using a tissue processor (Leica). The tissues were sectioned to 5 microns (Leica RM2255) and stained with hematoxylin and eosin (H&E) following the same protocol as Foothills Medical Centre Calgary Laboratory Services.

Statistical analysis

For survival plots, the log-rank Mantel-Cox test was used. For categorical variables, a two-way ANOVA with Bonferroni's multiple comparisons test was used. The development of all graphs as well as statistical analysis was performed using GraphPad Prism version 8.2.1.

Results

Intratumoral STING activation induces durable survival in UPS-bearing mice

DMXAA is an established murine-specific STING agonist with known dosing parameters and minimal toxicities below 30 mg/kg (28, 37). We first sought to determine if different dosing schedules of DMXAA would result in therapeutic anti-tumor effects. Single, double (3 days apart) or triple (every three days) i.t. doses of DMXAA resulted in complete tumor eradication beyond 3 months in 50-60% of mice (Figure 1A). I.t. dosing of DMXAA was chosen over intra-peritoneal administration to maximize local induction of i.t. immune responses. Additionally, there are reports that i.t. DMXAA is more effective at activating STING responsiveness in tumors than i.p. administration (28). We observed greatest tumor eradication in the triple dosed cohort but did observe overlying skin necrosis in over 50% of these mice. There we no observed toxicities in the 18 mg/kg

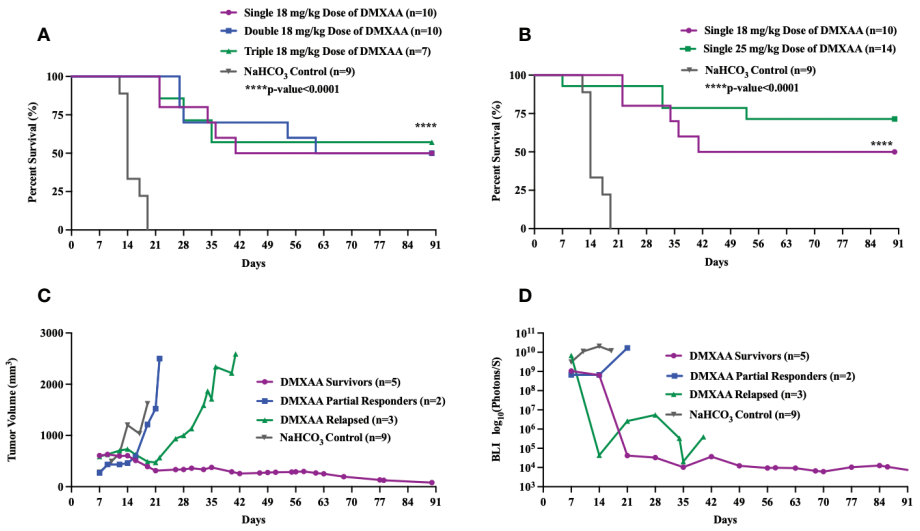

FIGURE 1
Intratumoral STING activation results in long-term survival in UPS-bearing C57Bl/6 mice. **(A-D)** 100,000 UPS + mCherry and luciferase cells were injected intramuscularly on day 0. **(A)** DMXAA (18 mg/kg) was injected i.t. according to varying dose schedules: single dose = injected on day 7, double dose = injected on days 7 and 14, and triple dose = injected on days 7, 11, and 14. **(B)** DMXAA (18 mg/kg or 25 mg/kg) or $NaHCO_3$ was injected i.t. on day 7. Mean tumor volume **(C)** and mean BLI **(D)** of UPS-bearing C57Bl/6 mice treated with DMXAA. **(C, D)** 18 mg/kg DMXAA or $NaHCO_3$ was injected i.t. on day 7.

group. Using a 25 mg/kg single i.t. dose we observed increased complete tumor eradication (70%) compared to the 18mg/kg dose (50%), although one mouse died from presumed treatment toxicity within 24hrs of injection (Figure 1B).

All DMXAA treated tumors showed immediate tumor volume and BLI reductions compared to control. A more detailed examination of tumor volumes and tumor BLI data in the single 18 mg/kg treated mice shows three distinct patterns of response to DMXAA treatment: long-term survivors, partial responders, and late relapse (Figures 1C, D). In the partial responder group, the mean tumor volumes steadily increased after a transient reduction (Figures 1C, D). In the relapsed group, mean tumor volume and BLI signal steadily decreased, and the tumors were no longer palpable, however around day 28, tumors became palpable again with associated increased BLI signal (Figures 1C, D).

UPS re-challenge is rejected in STING-treated surviving mice

We next sought to determine if successful clearance of UPS tumors following STING therapy would result in systemic protection against UPS recurrence. To mimic the clinical scenario of sarcoma recurrence in the extremity (local) or lung (metastatic), we performed UPS re-challenge experiments on previous UPS-bearing mice that completed eradicated their tumors after STING therapy. "Survivor" mice were re-challenged with UPS cells in either the contralateral limb or lung resulted and 100% of these mice rejected the UPS re-challenge as defined by no BLI signal or palpable tumor for up to 60 days of observation (Figure 2). All control mice in these experiments developed hindlimb and lung tumors that rapidly progressed to humane endpoint (Figure 2). There were no differences in UPS tumor clearance between males and females (Figure S1).

STING activation in extremity UPS tumors results in systemic clearance of limb tumors and synchronous lung lesions

The lung is the most common site of metastases in STS. To evaluate if STING treatment of extremity UPS tumors could also induce therapeutic responses in sites of distant disease, we tested STING activation in a model of synchronous hindlimb and lung tumors. Naïve mice were engrafted with UPS cells in the lung *via* tail vein injections (Day 0), followed by UPS engraftment in the right hindlimb (Day 7), and then given DMXAA (Day 14; 18 mg/kg) i.t. (Figure 3A). All mice developed engrafted UPS tumors in the lung and hindlimb as detected by BLI imaging. Mice bearing simultaneous hindlimb and lung UPS tumors that received i.t. STING therapy all survived longer than control

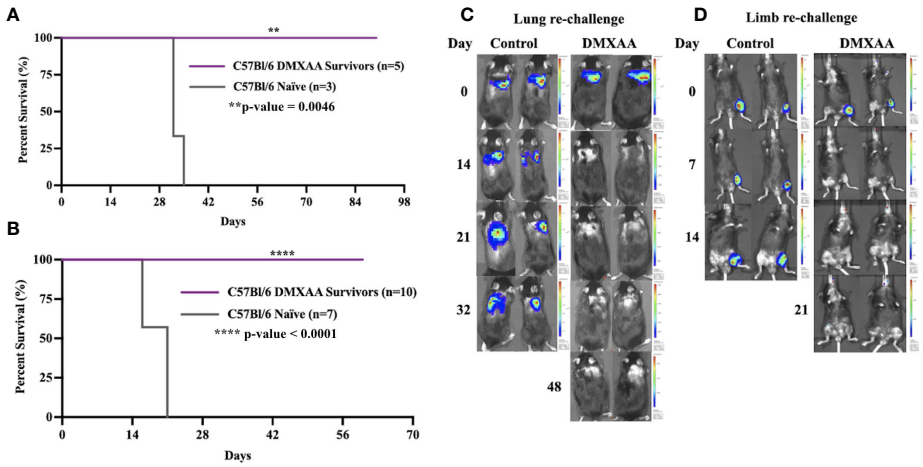

FIGURE 2
Intratumoral STING activation provides protective immunity against UPS re-challenge. **(A, C)** C57Bl/6 DMXAA survivors and naïve C57Bl/6 mice were given 100,000 UPS TAO1+ mCherry and luciferase cells injected *via* tail vein on day 0. **(B, D)** "Survivors" and naïve C57Bl/6 mice were re-challenged with 10,000 UPS TAO1+ cells injected intramuscularly into the contralateral limb on day 0. BLI images of tail vein re-challenge **(C)** and contralateral limb re-challenge **(D)** in naïve C57Bl/6 mice (control) and DMXAA survivors (DMXAA). **p-value=0.0046. ****p-value<0.0001.

mice, with 30% of STING treated mice completely eradicating UPS tumors in both anatomic sites (Figure 3B). By day 49, all surviving mice had complete and durable tumor remission in both sites (Figures 3C, D). Examining individual BLI data, 50% of mice that did not survive STING therapy developed severe tumor burden in the lung, and similar to isolated hindlimb DMXAA experiments, some mice transiently cleared the lung tumors only to relapse around 3 weeks post-therapy (Figure 3E).

Intratumoral STING activation results in tumor necrosis, lymphocyte infiltration, and upregulation cytotoxic adaptive immune pathways

To elucidate the changes within the UPS TIME following STING therapy, ex-vivo analyses of DMXAA treated UPS tumors were evaluated at multiple time points after treatment. Mid-tumor H&E sections showed >50% necrosis in all DMXAA treated tumors at 72hrs, with minimal spontaneous necrosis in control tumors (Figures 4A, B). Transcriptomic analyses also demonstrated higher apoptotic pathway scores was at 72hrs post STING treatment compared to control (Figure 4C).

Using FACS and Nanostring® transcriptome analyses, we sought to evaluate changes in immune populations within UPS tumors following STING treatment at various timepoints. Overall leukocyte infiltration and general inflammation scores were increased within 72hrs of STING treatment (Figures 4D, E). Additionally, there was elevated mRNA expression of downstream markers associated with the STING pathway or effectors of STING activation (Figure 4F), such as *Tbk1*, *Irf3*, as well as interferons alpha-1, 2, and 4 (*Ifna1, 2, and 4*), beta-1 (*Ifnb1*), and gamma receptor (*Ifngr*), thus further confirming evidence of persistent upregulation of STING pathway and effectors up to 72hrs following DMXAA treatment.

Assessing the myeloid immune compartment, gene expression levels of most macrophage markers were decreased at early time points post STING therapy but rebounded and were elevated relative to control by 72hrs (Figure 4G). Mean macrophage function scores were also increased 72hrs post STING treatment compared to control UPS tumors (Figure 4H). Using FACS we observed a rapid increase in neutrophils at early timepoints following STING treatment, which like the mRNA analyses, was associated with a reciprocal reduction in macrophages as well. This trend, however, was reversed by 7 days, where macrophage numbers steadily increased and neutrophil numbers declined (Figure 4I).

Examining the adaptive immune compartment, STING treated tumors demonstrated an elevation in adaptive immune scoring of mRNA expression profiles 72hrs after treatment (Figure 5A). T cell function scores and cytotoxic scores of mRNA analytes were also elevated in the 72hrs post DMXAA treatment group (Figures 5B, C). Direct mRNA expression levels of common lymphocyte markers were most upregulated in the 72hrs post DMXAA treatment in UPS tumors when compared to the control UPS tumors and 24hrs post DMXAA treatment

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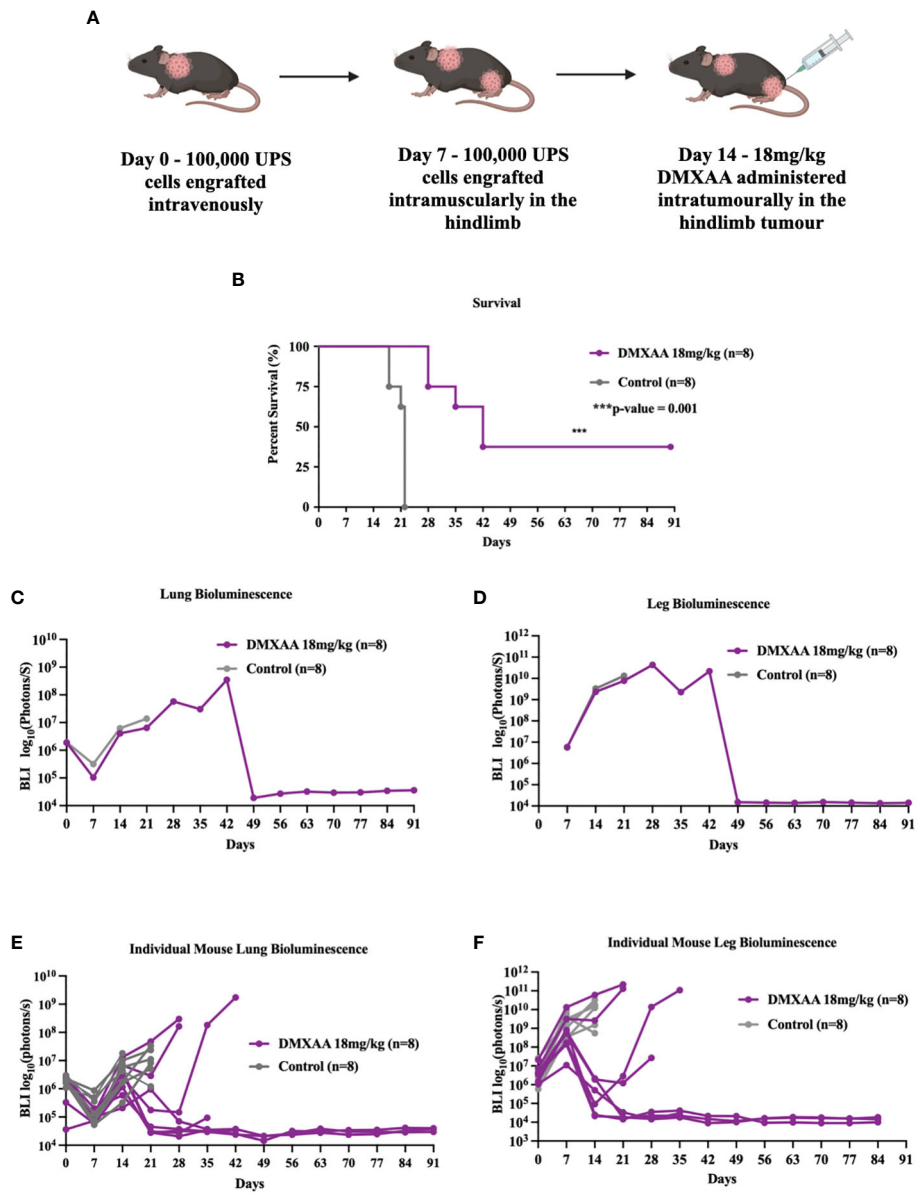

FIGURE 3
STING treatment of extremity UPS results in systemic eradication of synchronous lung metastases. **(A)** A schematic outlining the establishment of UPS tumors in the lung on day 0. 100,000 UPS TAO1+ mCherry and luciferase cells were injected in the tail vein, subsequently followed by leg tumor engraftment on day 7, and treatment of the hindlimb tumors with 18 mg/kg of DMXAA or vehicle control sodium bicarbonate i.t. on day 14. **(B)** Kaplan Meier plot comparing the survival of DMXAA, and vehicle control treated mice. **(C, D)** BLI intensity of leg and lung tumors in DMXAA and vehicle control groups. **(E, F)** BLI intensity of leg and lung tumors in DMXAA and vehicle control tumors individually.

(Figure 5D). There was a higher expression of *Cd3ϵ*, *Cd4*, and *Cd8* in control and tumors 72hrs after DMXAA treatment compared to 24hrs after treatment. However, there was an elevated expression of cytotoxic markers (Granzymes A and B; *Gzma* and *Gzmb*, Figure D) in tumors 72hrs after DMXAA treatment. Using FACS, compared to control, increased ratios of CD8+ T-cells were also observed in the STING treated UPS tumors seven days after treatment, while the quantity of CD4+ T-cells remained stable across all time points (Figure 5E).

Collectively, these investigations of the UPS TIME demonstrate that i.t. STING activation results in tumor necrosis, liberation of STING effector chemokines and cytokines, early neutrophil influx, followed by increases in adaptive immunity gene expression and CD8+ lymphocyte infiltration.

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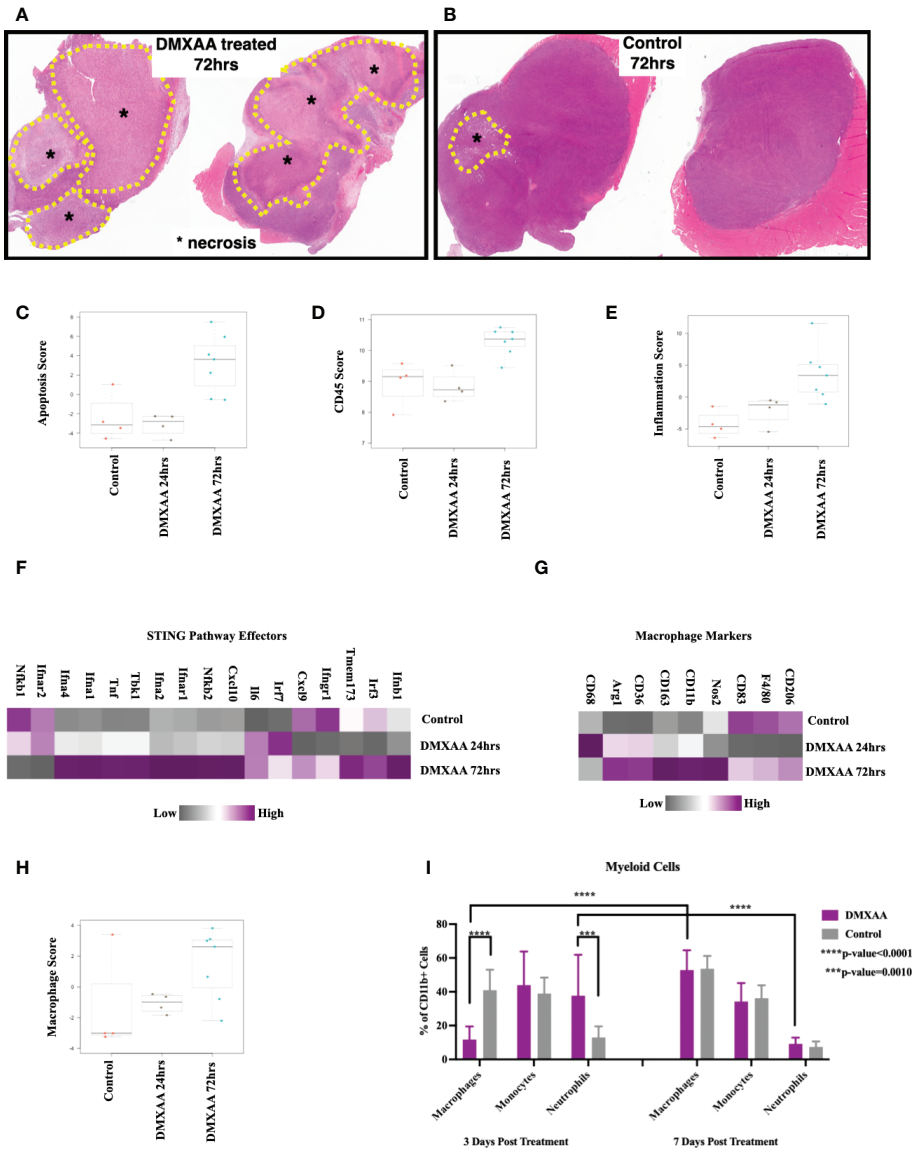

FIGURE 4
Intratumoral STING activation results in necrosis and upregulation of apoptotic and myeloid markers. **(A, B)** Low magnification microscopy of hematoxylin and eosin (H&E) stained tumor mid-sections shows substantial tumor necrosis 72hrs post STING therapy in UPS tumors. Nanostring nSolver® analyses of immune mRNA transcripts demonstrating increased apoptosis **(C)**, leukocyte infiltration **(D)**, and tumor inflammation **(E)** within 72hrs of STING therapy. nSolver® generated heatmaps show increased mRNA expression profiles of common STING pathway and effectors markers **(F)**, macrophage markers **(G)**, and macrophage functional scores **(H)** 72hrs after STING therapy. **(I)** FACS analyses of tumor cell suspensions for myeloid cells (CD45+, CD11b+), which includes macrophages (Ly6G-, Ly6C-), monocytes (Ly6G-, Ly6C+), and neutrophils (Ly6G+, Ly6C+).

Lymphocyte deficiency, but not macrophage deficiency, attenuates anti-tumor benefits of intratumoral STING immunotherapy

To determine if STING-mediated tumor clearance is dependent on an adaptive immune response, we tested DMXAA treatment in Rag2 Knockout (KO) mice (Figure 6). UPS engraftment, growth kinetics and time to humane endpoint were unaffected by lymphocyte deficiency (Figures 6A, B). There was also no significant difference between the overall survival time (p-value = 0.1728) of UPS bearing Rag2 KO mice and C57Bl/6 mice (Figure 6C). These findings suggest negligible engagement of the adaptive immune compartment in the progression of tumor growth or engraftment in this UPS model. The anti-tumor effects of STING therapy, however, were lost when UPS engrafted Rag 2

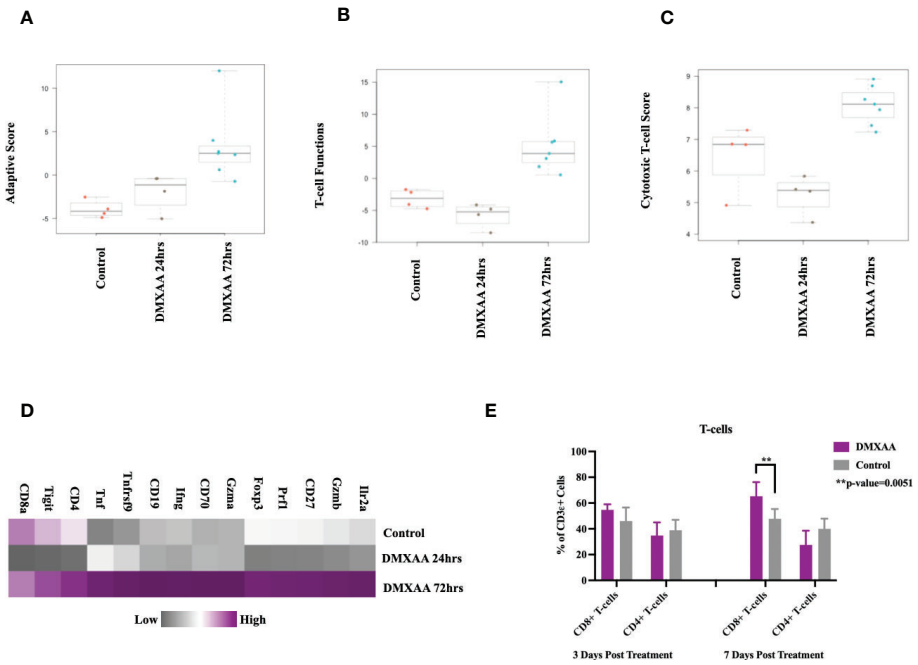

FIGURE 5
Intratumoral STING activation results in upregulation of lymphocytic markers and infiltration of cytotoxic T-lymphocytes. nSolver® advanced analysis of STING treated of UPS tumors demonstrates increased **(A)** adaptive immune pathway scores, **(B)** T-cell function scores, and **(C)** cytotoxic T-lymphocyte scores. Heat maps illustrate the **(D)** increased adaptive and cytotoxic mRNA expression profiles observed in UPS tumors following STING treatment. **(E)** FACS analyses of tumor cell suspensions assessing CD8 T cells (CD3ε+, CD8+) and CD4 T cells (CD3ε+, CD4+).

KO mice were treated with intra-tumoral DMXAA (Figures 6C-E). A marked decrease in UPS tumor volume was observed in DMXAA treated Rag2 KO mice (days 7-14; Figure 6C), with tumor volumes sharply rebounding afterwards. These observations would suggest early tumor clearance following STING therapy *via* lymphocyte independent mechanisms, although UPS tumors could not be cleared beyond 14 days without an intact lymphocyte compartment.

As STS are highly enriched in macrophages and given that macrophages are highly responsive to STING agonists (37–39), we sought to determine if reductions in UPS macrophages would mitigate the early or innate immune response to DMXAA. The CCR2/CCL2 is a known recruitment axis for tumor associated macrophages and highly expressed by TAO1 cells in culture (Figure 7A) we utilized a CCR2 KO model, which leads to deficiencies in monocyte recruitment into tumors (33) and has been shown reduced tumor macrophages in previous work (40). Engrafted UPS tumors in CCR2 KO mice showed 75% reduction of macrophages in UPS tumors (Figure 7B), but no differences in tumor growth kinetics and time to humane endpoints (Figures 7C, D). Following i.t. DMXAA, both control and CCR2 KO mice showed reduction in UPS tumor volumes (Figures 7E), tumor bioluminescence (Figure 7F), and tumor free survival 90-days post-UPS engraftment (Figure 7G). However, UPS tumors in the CCR2 KO group demonstrated quicker tumor volume and BLI response to treatment (Figures 7E, F). These results would suggest that tumor macrophage reductions *via* the CCR2/CCL2 axis did not impair responsiveness to STING agonist therapy and may have promoted a more rapid early/innate response.

STING therapy is synergistic with immune checkpoint blockade in murine UPS

This murine model of UPS is resistant to anti-CTLA4 and anti-PD-1 monotherapy (Figures 8A, B) and documented by others (29). We have observed late UPS tumor relapses in mice treated with DMXAA after near complete tumor eradication (relapses, Figures 1C, D). As we have also observed increased CD8+ T cell infiltration and cytotoxic lymphocyte scores following STING treatment of UPS tumors, we sought to determine genes associated with negative immune regulation were upregulated in UPS tumors after i.t. DMXAA. We observed upregulation of *Ido1*, *Lag3*, *Pd-1*, *Ctla4*, *Pdcd1lg2*, and *Tigit*

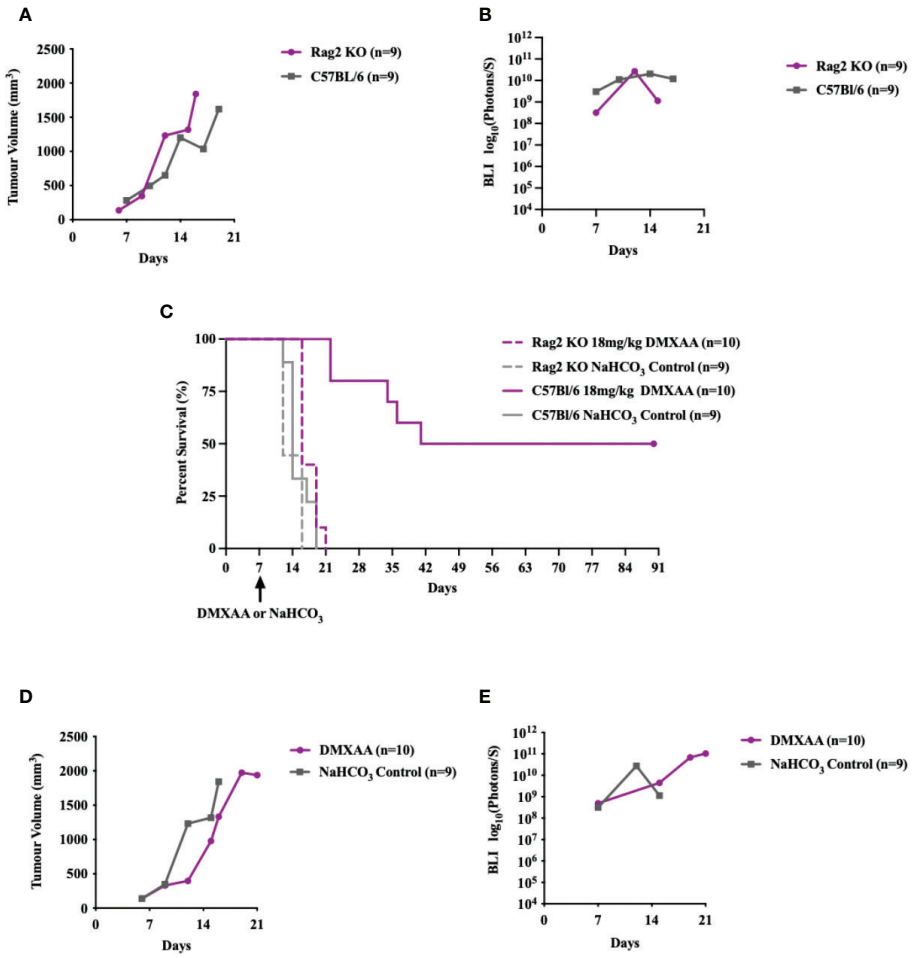

FIGURE 6
Intratumoral STING activation and subsequent anti-UPS tumor effects are mediated by adaptive immune responses. **(A-E)** 100,000 UPS + mCherry and luciferase cells were injected intramuscularly on day 0. **(A)** Mean tumor volume and **(B)**–mean BLI ROI of UPS growth in Rag2 KO mice (purple) and C57Bl/6 mice (grey). **(C)** Survival of untreated Rag2 KO (solid purple) and C57Bl/6 mice (solid grey), as well as Rag2 KO (dashed purple) and C57Bl/6 mice (dashed grey) treated i.t. with DMXAA (18 mg/kg) on day 7. **(D)** Mean tumor volume and **(E)** mean BLI ROI of Rag2 KO mice (purple) and C57Bl/6 mice (grey) treated i.t. with DMXAA (18 mg/kg) on day 7.

transcripts 72hrs post DMXAA treatment compared to control UPS tumors (Figure 8C). Mean exhausted CD8 scores were also at this timepoint (Figure 8D), collectively implying an opportunity to increase therapeutic outcomes in STING treated UPS tumors by the addition of immune checkpoint inhibition (ICI) therapy.

The additional of both anti-PD1 and anti-CTLA4 therapy improved STING-mediated tumor clearance from 50% to 80%. We also observed 30% tumor clearance using combination ICI therapy without STING therapy in this UPS model (Figure 8E). These results suggest (i) there is baseline negative immune checkpoint regulation in this model that can be therapeutically targeted using combination ICI therapy, but not monotherapy and (ii) STING activation results in further upregulation of negative T cell co-stimulatory pathways that can be targeted to improve tumor clearance.

Discussion

Soft tissue sarcomas (STS) are rare, high-fatality cancers that are poorly responsive to systemic therapies (6, 41–44). Recent clinical trials have persistently failed to show significant clinical benefit for patients with advanced STS treated with immune checkpoint inhibitors (6, 12, 45), and other immune-based therapies (7, 9, 46–50). While considerable heterogeneity exists within the complex karyotypes of STS, the TIME of most STS is immunologically cold, which predicts poor sensitivity to immune

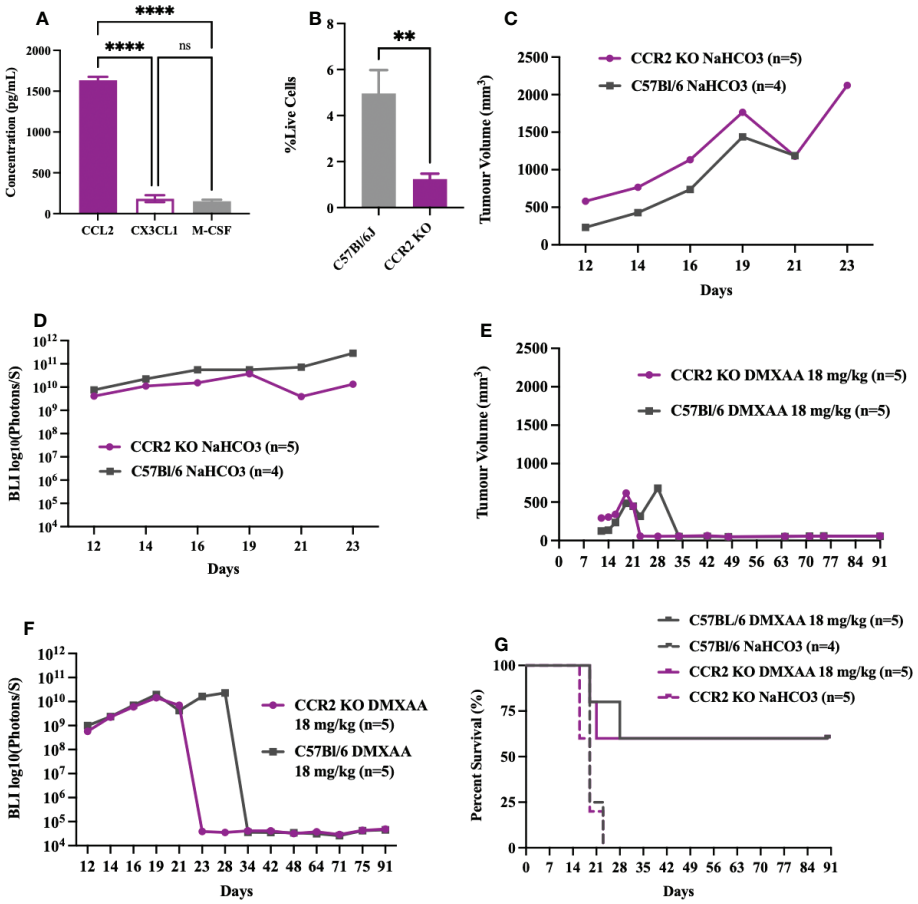

FIGURE 7
Impairing monocyte recruitment with CCR2 deficiency showed similar UPS response to intratumoral STING activation. **(A)** Concentration of monocyte chemoattractants (CCL2, CX3CL1, M-CSF) in the supernatant of UPS cell culture. **(B-G)** 100,000 UPS + mCherry and luciferase cells were injected intramuscularly on day 0. **(B)** UPS tumor macrophages (CD45+, CD11b+, F4/80+) in CCR2 KO mice are reduced by 75% compared to control C57Bl/6 mice 9-days after UPS engraftment. **(C)** Mean tumor volume and **(D)** mean BLI ROI of UPS tumor growth curves in CCR2 KO mice and C57Bl/6 mice. **(E)** Tumor volume and **(F)** mean BLI ROI of UPS-bearing CCR2 KO or C57Bl/6 mice treated with 18 mg/kg DMXAA i.t. on day 7. **(G)** Longitudinal *in vivo* survival of UPS bearing mice following STING therapy showing similar overall survival in CCR2 KO and control C57Bl/6 mice. ns, non-significant. **p-value=0.043. ****p-value<0.0001

therapies (22). Using a transplantable, immune competent, orthotopic murine model of UPS that recapitulates the lymphocyte poor TIME of most STS, we sought to determine if STING immunotherapy could dismantle the immunosuppressive features of this model and promote immunogenic tumor eradication. Here, we demonstrate that i.t. STING activation can promote tumor necrosis, lymphocyte mediated tumor clearance and durable tumor eradication in up to 60% of UPS-bearing mice following a single injection of a small molecule STING agonist. Additionally, i.t. STING therapy was also effective on systemic sites of disease in the lung, and in mice that cleared tumor following therapy, durable immunity against UPS re-challenge was present.

While there have been numerous studies examining the therapeutic potential of STING agonism in solid tumor models (19, 28, 51–53), this is the first detailed examination of STING therapy in an immunologically cold model of sarcoma. Recently, Wolf et al., did test a STING agonist, ADU-S100, in combination with an IL-2 superkine (H9-MSA) using the methylcholanthrene carcinogen model of sarcoma (51). This model of UPS has a high mutational burden (2000 non-synonymous mutations/tumor) compared to the KP UPS model (18 non-synonymous mutations/tumor) and is more representative of the mutational burden observed in human cancers that are sensitive to immunotherapies (54, 55). Conversely, the TIME of the KP model of UPS contains a paucity of lymphocytes, is enriched in

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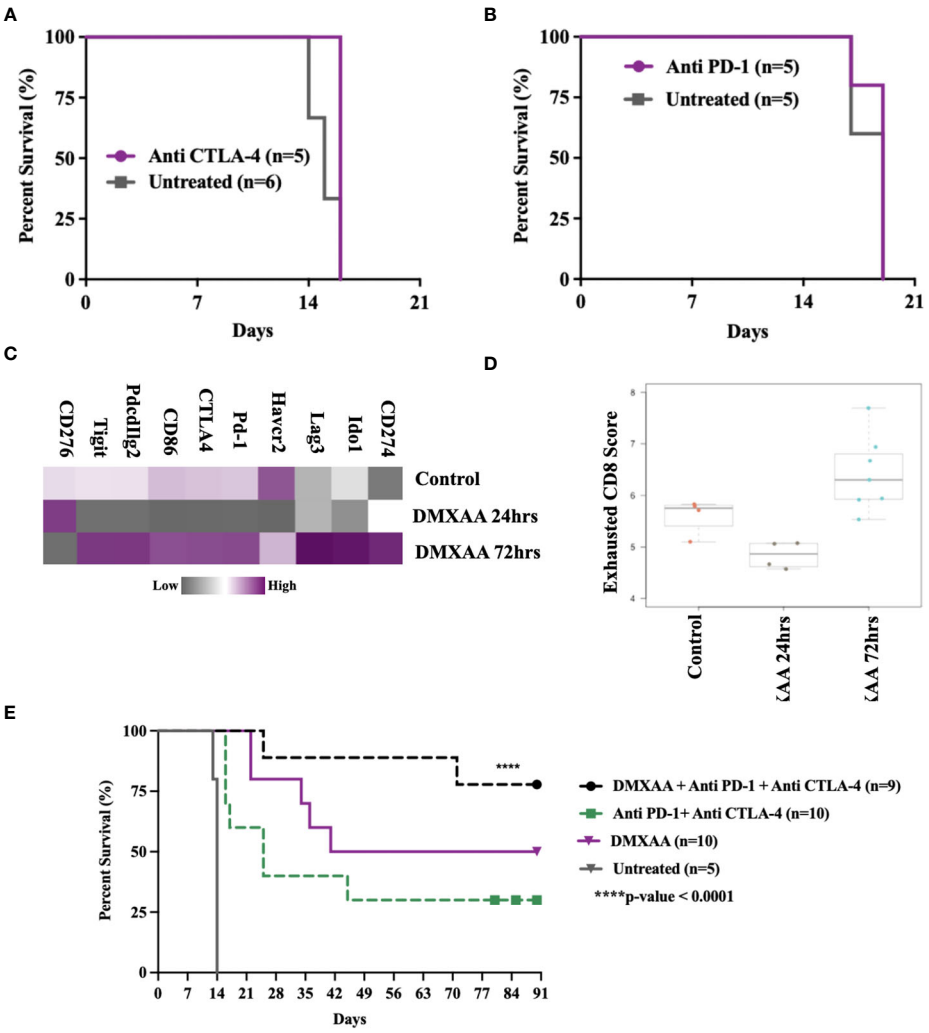

FIGURE 8
Therapeutic synergy of STING activation and immune checkpoint blockade in murine UPS tumors. **(A, B)** 100,000 UPS + mCherry and luciferase cells were injected intramuscularly on day 0. **(A)** 250 µg mouse anti-CTLA4 monoclonal antibody or **(B)** 250 µg mouse anti-PD1 monoclonal antibody were injected intraperitoneally, on days 7, 10, and 13 following UPS engraftment. **(C)** NanoString mRNA expression profile of common immune checkpoint markers. Upregulated expression is shown in purple and downregulated expression is shown in grey. Control UPS tumors (n=4), 24 hours post DMXAA UPS tumors (n=4), 72 hours post DMXAA UPS tumors (n=7). **(D)** Exhausted CD8 pathway score using Nanostring Technologies.**(E)** Anti-PD-1 (250 µg) + anti-CTLA4 (250 µg) were injected intraperitoneally on days 9, 11, 15, 18, 22, 25, 29, and 32 following UPS injection (black and green), DMXAA (18 mg/kg) was injected i.t. on day 7 (purple).

CD206 immunosuppressive macrophages, and is resistant to immune checkpoint blockade (29, 56), thus recapitulating the immunotherapy resistant phenotype common to most sarcomas. We do recognize that our UPS model used here is driven by Kras and p53 mutations, which are also used to induce lung and pancreatic carcinomas in mice. Indeed, there is also evidence that STING activation can induce therapeutic responses in these models (37, 57), suggesting that these mutations or associated downstream effector pathways may support sensitivity to STING therapy.

Similar to other studies, we have shown that STING-mediated clearance of tumor cells in this UPS model is dependent on functional lymphocytes (28, 52, 53). Additionally, our data importantly shows the resultant systemic treatment effect following i.t. STING activation as we observed durable survival in mice with synchronous extremity and lung tumors following treatment of the extremity tumor. This, coupled to the rejection of UPS re-challenge in the leg or lung highlights the persistent anti-sarcoma systemic adaptive immunity following a single treatment of STING activation. This is clinically important as the lung is the

principal visceral site of STS metastases or systemic relapse (58, 59). These data justify further study into how STING-based immunotherapy for primary sarcomas could be used to systemically eradicate micro-metastases or prevent relapses following local control procedures.

A central process of STING immunotherapy is the induced cooperation of rapid innate immune responses with persistent adaptive immune-based elimination of cancer cells. Numerous studies have demonstrated impaired STING signaling or downregulation of STING in cancer cells, suggesting the stromal constituents of the TIME are the critical targets of STING activation (60–63). As macrophages are highly sensitive to STING agonists (64–66) and are abundant in both human and pre-clinical sarcoma models (29, 39, 56, 67–69), we hypothesized that a reduction in tumor associated macrophages (TAMs) would mitigate the therapeutic response to STING agonism. Monocytes are known to contribute to the TAM populations, and the CCR2/CCL2 signaling is critical for TAM recruitment from monocyte lineages (55). We did observe a 75% reduction in UPS TAMs in the CCR2 KO line but did not observe any change in long-term survival and instead observed earlier onset of tumor volume and BLI reductions following treatment. It is possible TAMs are not the dominant effector cell of small molecule STING agonists in this model and STING signaling occurs *via* other cell populations such as tumor resident DCs (21), endothelial cells (19) or remaining macrophage pools. Alternatively, inhibition of the CCL2/CCR2 axis is associated a decrease in CD206 immunosuppressive macrophage populations (38) and thus a reduction in CD206 TAMs in CCR2 KO mice may provide a more inflamed and sensitive environment for STING responsiveness.

An interesting observation in these experiments were the late tumor relapses following STING therapy. In these mice (30%), tumors substantially regressed and were not palpable, but quickly rebounded 2-3 weeks after treatment. Transcriptomic data of STING treated tumors did show increased expression of markers associated with T-cell inhibition and T-cell exhaustion which could explain late treatment resistance. Supporting this, we observed improved tumor clearance from 50% to 80% when STING therapy was combined with immune checkpoint inhibition (anti-CTLA4 and anti-PD1). These observations are consistent with pre-clinical studies in other cancer models showing STING-dependent upregulation of negative immune checkpoints and improved therapeutic responses when STING agonism is combined with immune checkpoint blockade (37, 52, 70). As there is considerable clinical enthusiasm to understand which clinical STS will benefit from immune checkpoint blockade, the addition of intra-tumoral STING therapy may provide an opportunity to improve response rates across more STS subtypes.

We acknowledge that there are limitations within the present study. Firstly, it has been well characterized that DMXAA is murine-specific STING agonist and does not activate human STING (71). We elected to use DMXAA in this study as proof of concept given the documented efficacy, known toxicities and well-defined dosing parameters of this small molecule STING agonist. Over the past decade, numerous small molecule STING agonists capable of activating human STING have been developed (51, 52, 72) and while some of these agents are now being tested in clinical trials, there remains much to learn regarding how these new agents should be administered and dosed locally, systemically, and in concert with other therapies. Future studies are ongoing evaluating these new agents in different genetic models of STS. Another limitation pertains to the cell-line derived UPS tumors used in this study. We and others have shown that engrafted KP UPS tumors demonstrate increased spontaneous lymphocytic infiltrated compared to spontaneous KP UPS tumors (56). Therefore, these engrafted tumors may be more sensitive to STING therapy and future studies evaluating spontaneous tumors will be required. Engraftable tumors enabled a more consistent, reproducible, and feasible experiments as we could predictably induce tumors and begin therapy using consistent timelines. Further work will be completed to delineate the tumor antigens involved in this UPS model following STING activation.

Conclusion

To our knowledge, this is the first study to evaluate STING immunotherapy in the KP model of UPS. Like most human STS, the KP sarcoma model has an immune-suppressed TIME and is resistant to immune checkpoint blockade. We have shown that a single treatment of intra-tumoral STING activation can induce immune-mediated sarcoma clearance locally and systemically. These results justify further study into the clinical translation of STING immunotherapy for sarcomas.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by University of Calgary Health Sciences Animal Care Committee (#AC19-0072).

Author contributions

KLM contributed to project conception, completion, study design, and produced the first draft of the manuscript. KMH and KNH contributed to experiment completion and final manuscript production. AKS contributed to study design and experiment execution. FJZ, DJM, and FRJ contributed to study conception and project design. MJM contributed to project conception, design, manuscript development, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1087991/full#supplementary-material

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Emerging targeted and cellular therapies in the treatment of advanced and metastatic synovial sarcoma

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Synovial sarcoma is a soft tissue sarcoma accounting for approximately 1,000 cases per year in the United States. Currently, standard treatment of advanced and metastatic synovial sarcoma is anthracycline-based chemotherapy. While advanced synovial sarcoma is more responsive to chemotherapy compared to other soft tissue sarcomas, survival rates are poor, with a median survival time of less than 18 months. Enhanced understanding of tumor antigen expression and molecular mechanisms behind synovial sarcoma provide potential targets for treatment. Adoptive Cell Transfer using engineered T-cell receptors is in clinical trials for treatment of synovial sarcoma, specifically targeting New York esophageal squamous cell carcinoma-1 (NY-ESO-1), preferentially expressed antigen in melanoma (PRAME), and melanoma antigen-A4 (MAGE-A4). In this review, we explore the opportunities and challenges of these treatments. We also describe artificial adjuvant vector cells (aAVCs) and BRD9 inhibitors, two additional potential targets for treatment of advanced synovial sarcoma. This review demonstrates the progress that has been made in treatment of synovial sarcoma and highlights the future study and qualification needed to implement these technologies as standard of care.

KEYWORDS

synovial sarcoma, soft tissue sarcoma, therapeutics, clinical trial, adoptive cell transfer

1 Introduction

Synovial sarcoma (SYN) is a soft tissue sarcoma accounting for 5-14% of all soft tissue sarcomas (1, 2). The incidence of SYN in the United States is approximately 1.42 per million for adults and 0.81 per million for children and adolescents, accounting for roughly 1,000 cases per year (3). SYN presents at an average age of 35-40 years and there is equal distribution of cases between females and males (3–6). SYN most often arises in deep tissues

of the extremities but can also present as head and neck, trunk, and lung lesions (3, 7). Epidemiologic studies have found that most patients are diagnosed with local disease while 10-13% of patients initially present with metastatic disease (3, 7).

The diagnosis and staging of SYN involve pathologic and radiographic review. SYN is defined by the presence of translocation of t(X:18) (p11.2;q11.2) using FISH or RT-PCR and is found in more than 95% of tumors (8). This translocation leads to the fusion of genes *SYT* on Chromosome 18 and *SSX* on Chromosome X, which causes production of SS18-SSX1, SS18-SSX2, or SS18-SSX4 (9–11). These oncogenic fusion proteins impact cellular transcription and metabolism, leading to sarcomagenesis.

For localized cases of SYN, initial therapy is most commonly surgical resection with or without radiation therapy. Neoadjuvant or adjuvant chemotherapy is considered in select cases (12, 13). SYN has high metastatic potential with a historic five year metastasis-free survival rate of 50-60% (14). For locally advanced or metastatic disease, first line therapy usually incorporates anthracycline-based chemotherapy with or without ifosfamide (13, 15, 16). SYNs are relatively chemosensitive tumors compared to other soft tissue sarcomas. In primary soft tissue sarcomas, early localized and metastatic recurrence have been found to occur at a median of 38.3 and 41.3 months, respectively (17). In contrast, SYN has been found to have local recurrence at a mean of 43 month and metastatic recurrence at 68 months (18). A review of 15 clinical trials of first-line chemotherapy for SYN has shown a 27.8% response rate compared to 18.8% in other soft tissue sarcomas (19). When comparing SYN to other soft tissue sarcomas, progression free survival (PFS) was 6.3 months versus 3.7 months and overall survival (OS) was 15.0 months versus 11.7 months, respectively (19). Despite this response, however, for those with metastatic disease one year survival remains 59.5% and the median overall survival is 17.0 months (95% CI 14.5-19.5) (6).

Currently, after anthracycline-based chemotherapy, the only other systemic therapy for treatment of advanced or metastatic SYN approved by the FDA is pazopanib. This approval was granted after pazopanib was shown to improve PFS compared to placebo in a population of patients with varying non-adipocytic metastatic soft tissue sarcomas which included 30 patients with SYN (20).

Improved understanding of cellular and molecular processes behind the development of SYN and advancements in knowledge of SYN's antigen expression will allow for potential targets for treatment of advanced SYN. In this review, we explore emerging therapies in the treatment of advanced and metastatic SYN.

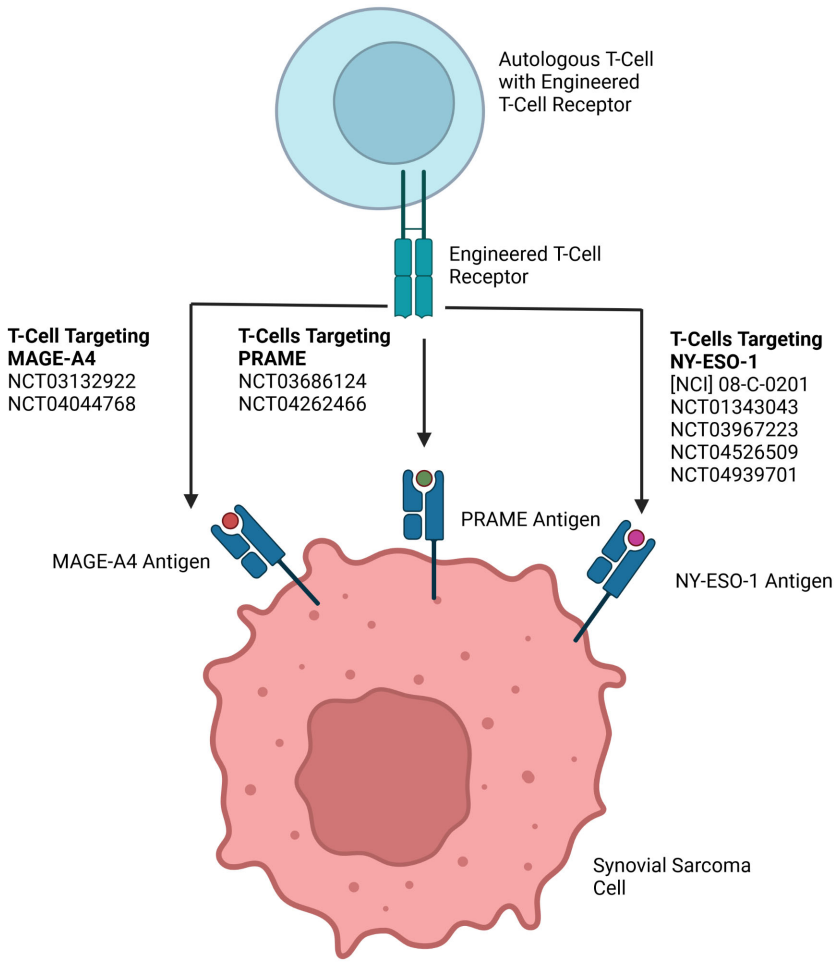

FIGURE 1
Adopted Cell Transfer engineered T-cell receptors targeting CTAs for treatment of SYN.

TABLE 1 Clinical trials completed or with preliminary results for treatment of SYN.

Target	Treatment, Population	Trial, Publication year	Phase, Study Size	Summary
NY-ESO-1	Anti-NY-ESO-1 T-cells, HLA- A*0201, Metastatic SYN or melanoma	[NCI] 08-C-0201, 2015 (30, 31)	II, 18 with SYN	1 complete response, 10 partial responses. Three-year survival 38%, five-year survival 14% AEs: 100% of patients with neutropenia and thrombocytopenia
	Autologous NY-ESO-1^{c259} T-cells, HLA- A*02, Unresectable, metastatic, or recurrent SYN	NCT01343043, 2020 (26, 32)	I, 45	1 complete response (34 weeks), 14 partial responses (across four cohorts) AEs: 40% with Grade 3 or higher hematologic AEs, 44% with cytokine release syndrome (4 patients Grade 3 or higher)
PRAME	Anti-PRAME T-cells, HLA matching, PRAME expression solid tumors	NCT03686124*, 2021 (33)	I, 12	6 patients with partial response, 6 with stable disease (3 patients with SYN) AEs: cytopenia, cytokine release syndrome and neurotoxicity (Grade 1-2), one dose limiting toxicity
MAGE-A4	Autologous MAGE-A4^{c1032} T-Cells, HLA-A*02, expression of MAGE-A4	NCT03132922, 2020 (34, 35)	I, 28	7 patients with partial response, 11 with stable disease AEs: No dose limiting toxicities, >30% Grade 3 or higher hematologic AEs, two trial related deaths due to aplastic anemia and cerebral vascular accident
	Anti-MAGE-A4 T-cells, HLA-A*02, Advanced SYN or myxoid/round cell liposarcoma	NCT04044768*, 2022 (36, 37)	II, 51	36.2% response rate, median duration or response 52 weeks (8.29 – 75.14) AEs: Not reported

*Preliminary results presented, recruitment continues.

2 SYN antigen expression and adopted cell transfer

SYN has been found to express cancer testis antigens (CTAs) (21). CTAs are antigens with predominant expression in the testis and are not normally found in somatic tissue (22). CTAs are a potential target for treatment of malignancies as they elicit humoral and cellular immune responses (23). SYN has been found to have high expression of CTAs (24). Due to their high expressivity, selectivity, and immunologic response, CTAs have been identified as potential targets for treatment of SYN.

Adopted Cell Transfer (or Therapy) (ACT) uses tumor antigen specific T-cells obtained from resected tumor specimens which are expanded *in vitro* and then infused for treatment of cancers (25, 26). One challenge of this treatment is that not all resected tumors allow for the expansion of autologous tumor infiltrating T-cells (27). This obstacle, along with variable quantities of T-cells within tumors, has prompted study of genetically engineered T-cell receptors to target cancer specific antigens. These T-cells are obtained through the harvesting of patient autologous T-cells which are then genetically modified to express a T-cell receptor for a cancer antigen. This technology is currently in development for the treatment of SYN targeting CTAs (Figure 1).

2.1 NY-ESO-1 adopted cell transfer

New York esophageal squamous cell carcinoma-1 (NY-ESO-1) is a CTA that was first described from serological analysis of recombinant cDNA expression libraries (SEREX) of esophageal squamous cell carcinoma (28). NY-ESO-1 is expressed in approximately 80% of SYNs. The immunogenicity of NY-ESO-1 has led to its consideration as a target for treatment of SYN (23, 29).

In 2011, Robbins et al. conduced the first clinical trial of autologous T-cells genetically engineered to have a specific T-cell receptor for NY-ESO-1 for patients with metastatic melanoma and SYN (Table 1) (38). The trial utilized a retroviral vector to create CD4+ and CD8+ autologous T-cells with a T-cell receptor that recognized the SLLMWITQC peptide of NY-ESO-1 for HLA-A*0201, named IG4- α95:LY ([NCI] 08-C-0121) (30). These T-cells were expanded *in vitro* and then transferred to patients after nonmyeloablative chemotherapy along with IL-2. This trial demonstrated objective clinical response in four out of six patients with SYN. The study was then expanded for 12 additional patients with SYN (31). Results demonstrated complete response for one patient and partial response observed in 10 of the 18 total patients with SYN. The three-year survival rate was 38% and the five-year survival rate was 14%.

All participants had neutropenia and thrombocytopenia during lymphodepleting chemotherapy, and one patient died from E. coli bacteremia three days after transfer of T-cells during a period of neutropenia. In this study, no correlation was measured demonstrating relationship between percentage of anti-NY-ESO-1 CD4+ or CD8+ T-cells at one month post transfer and disease response.

With evidence of activity for genetically engineered T-cells targeting NY-ESO-1, additional studies of genetically engineered T-cell receptors against NY-ESO-1 have been conducted. A Phase I, open-label trial of NY-ESO-1^{c259} T-cells (letetresgene autoleucel [lete-cel]; GSK3377794) included 45 patients with recurrent or metastatic SYN (NCT01343043) (29). This study resulted in one complete response (34

TABLE 2 Clinical trials in recruitment for treatment of SYN.

Target	Treatment	Population	Trial	Phase
NY-ESO-1	Autologous NY-ESO-1^{c259} T-cells	HLA- A*02, Previously untreated advanced SYN or myxoid/round cell liposarcoma	NCT03967223 (41)	II
	Anti-NY-ESO-1 T-cells with co-expression of CD8α chain Anti-NY-ESO-1 T-cells with co-expression of TGF-β Epigenetically reprogrammed NY-ESO-1 T-cells	HLA- A*02, Previously treated advanced SYN or myxoid/round cell liposarcoma	NCT04526509 (42)	I
	NY-ESO-1 aAVCs	Relapsed, refractory advanced solid tumors known to express NY-ESO-1	NCT04939701 (43)	I, II
PRAME	Anti-PRAME T-cells	HLA-A*02, Relapsed, refractory PRAME positive	NCT04262466 (44)	I, II
BRD9	BRD9 inhibitor (CFT8634)	Locally advanced or metastatic SMARCB1-perturbed cancers, including SYN	NCT05355753 (45)	I
	BRD9 inhibitor (FHD-609)	Advanced SYN or advanced SMARCB1-loss tumors	NCT04965753 (46)	I

weeks) and 14 partial responses (29, 32). In this trial, four cohorts were established with varying NY-ESO-1 expression and lymphodepleting chemotherapeutic regimens. Cohort 1 included patients with high NY-ESO-1 expression and a high lymphodepletion regimen (fludarabine and cyclophosphamide). Cohort 2 included patients with low NY-ESO-1 expression with a high lymphodepletion regimen. Cohort 3 included patients with high NY-ESO-1 expression with a differing high lymphodepletion regimen (cyclophosphamide only). Cohort 4 included patients with high NY-ESO-1 expression and a low lymphodepletion regimen (dose reduced cyclophosphamide and fludarabine).

Cohorts 1-3 have complete data available as of January 2020. In Cohort 1, six of 12 patients had at least a partial response, one patient had a complete response, and the median overall survival (OS) was 24.3 months (29). In Cohort 2, four patients of 13 had partial response and the median OS was 9.9 months. In Cohort 3, one patient of 5 had a partial response with an OS of 19.9 months. In Cohort 1 all six responders had presence of anti-NY-ESO-1 T-cells at 6 months post cells transfer (39). More than 40% of patients in all cohorts had Grade 3 or higher hematologic Adverse Events (AEs) and 44% of patients had cytokine release syndrome, of which four were Grade 3 or higher (29). This is similar to toxicity seen for chimeric antigen receptor (CAR) T-cell therapy, where 69% of patients had Grade 3 or higher neutropenia and 92% of patients had cytokine release syndrome, of which 6% were Grade 3 or higher (40). A Phase II master protocol is currently in recruitment to test NY-ESO-1 T-cells for patients with metastatic SYN or myxoid/round cell liposarcoma who have progressed after standard treatment (NCT03967223) (Table 2) (41).

Next generation NY-ESO-1 T-cell products may provide additional benefits, but qualification is needed. Currently, a Phase II master protocol of three different next generation NY-ESO-1 T-cell products is in recruitment for treatment of solid tumors with NY-ESO-1 expression (NCT04526509) (42).

CD8 is a cell surface glycoprotein that acts as a co-receptor with T-cell receptors and assists in T-cell binding to MHC1 (47, 48). Previous *in vitro* study has found that engineered T-cells targeting a cancer testis antigen that co-expressed CD8α led to greater CD4+ T-cell activity (49). One arm of the master protocol will use anti-NY-ESO-1 T-cells which co-express the CD8α chain to determine efficacy of this technology (GSK3901961) (42).

An additional technology of interest combines anti-NY-ESO-1 T-cells with a dominant negative transforming growth factor- β (TGF-β) type II receptor (GSK3845097) (42). TGF-β is a regulator of immune homeostasis and has been found to inhibit tumor cellular immunity (50, 51). T-cells genetically engineered to target prostate cancer combined with a dominant negative TGF-β receptor have been found to cause tumor regression and enhanced survival in a murine model (51). This technology may improve the tumor microenvironment by limiting the impact of immune down-regulators, specifically TGF- β, in treatment of SYN.

T-cell quality impacts success in ACT. Previous study of ex-vivo ACTs has found that stem-like surface markers on T-cells are more likely to lead to response and stem-like T-cells are more capable of *in vivo* expansion (52). This knowledge has led to the development of technology that improves the stem-like quality of engineered T-cell receptors ex vivo through epigenetic reprogramming (53). The third arm of the master protocol will assess anti-NY-ESO-1 T-cells after a proprietary epigenetic reprogramming process to enhance the stem-like quality of the T-cells (GSK4427296). Combining engineered T-cells with additional genetic modifications may enhance efficacy of ACT targeting NY-ESO-1. Beyond NY-ESO-1 targeted therapies, other ACTs against cancer testis antigens have been developed for the treatment of SYN.

2.2 PRAME adopted cell transfer

Preferentially expressed antigen in melanoma (PRAME) is a cancer testis antigen that is expressed in 95% of metastatic melanoma (54). It is also expressed homogenously in SYN at high levels (55). PRAME functions through inhibition of apoptosis and signal transduction of the retinoic acid receptor, causing tumorigenesis (56). Based on its expression and impact on sarcomagenesis, it is an additional target for directed engineered T-cell therapy.

The IMA203 trial utilized T- cell receptor engineered T-cells against PRAME in HLA-A*02:01 (NCT03686124) (57). This Phase I

trial of 12 evaluable patients resulted in six patients with stable disease and six patients with partial response, three of whom had SYN (33). The most common adverse events were cytopenias, neurotoxicity, and cytokine release syndrome. One patient had a dose limiting toxicity. Another, currently recruiting, trial for treatment of advanced solid tumors with PRAME and HLA-A*02:01 expression will test IMC-F106C, a T-cell receptor against PRAME, both in combination with checkpoint inhibitors and as a single agent (NCT04262466) (44). Results of this trial are expected in 2024.

2.3 MAGE adopted cell transfer

Melanoma-associated antigen (MAGE) proteins are clustered on the X chromosome. Expression of MAGE protein is generally restricted to reproductive tissues. This protein functions by inhibition of p53 and thereby limits tumor suppression (58, 59). MAGE-A4 is a cancer testis antigen that is expressed in many tumor types including lung cancer (19-35%), breast cancer (13%), ovarian cancer (47%), colon cancer (22%), esophageal cancer (60%), and soft tissue sarcomas, including 50-80% of SYN (24, 60–62).

Afamitresgene autoleucel are autologous T-cells which are isolated from patients, transduced with a lentiviral vector containing the MAGE-A4^{c1032} T-cell receptor, and expanded prior to infusion. Recently, results of a Phase I dose-escalation and expansion trial of Afamitresgene autoleucel was conducted in patients who were HLA-A*02 positive with advanced cancers that expressed MAGE-A4 (NCT03132922). In this study, patients received lymphodepletion regimen of cyclophosphamide and fludarabine prior to Afamitresgene autoleucel infusion (34, 35).

In the Cohort 3/expansion group (28 patients), 7 of 28 patients had a partial response, 11 of 28 had stable disease, while 10 of 28 either had progressive disease or were not evaluable (34). Results of this study showed no dose limiting toxicities and the most common Grade 3 or higher AE (>30%) were hematologic, including lymphopenia, leukopenia, neutropenia, anemia, and thrombocytopenia. Two patients had trial related deaths due to aplastic anemia and cerebral vascular accident. Notably, all responses to therapy occurred in patients with SYN, perhaps emphasizing the validity of targeting MAGE-A4 in this histology.

A Phase II, single arm, open-label clinical trial of Afamitresgene autoleucel in patients with advanced SYN or myxoid/round cell liposarcoma (MRCLS) called SPEARHEAD-1 is currently underway (NCT04044768) (36). Preliminary results from SPEARHEAD-1 were presented at the 2022 American Society of Clinical Oncology Annual Meeting (37). Patients received Afamitresgene autoleucel and were evaluable for response (Phase I, n = 18; Phase II, n = 51) with all patients expressing the HLA-A*02 allele. The pooled investigator-assessed overall response rate was 36.2% which occurred across MAGE-A4 H-scores of 134-400. The median duration of response

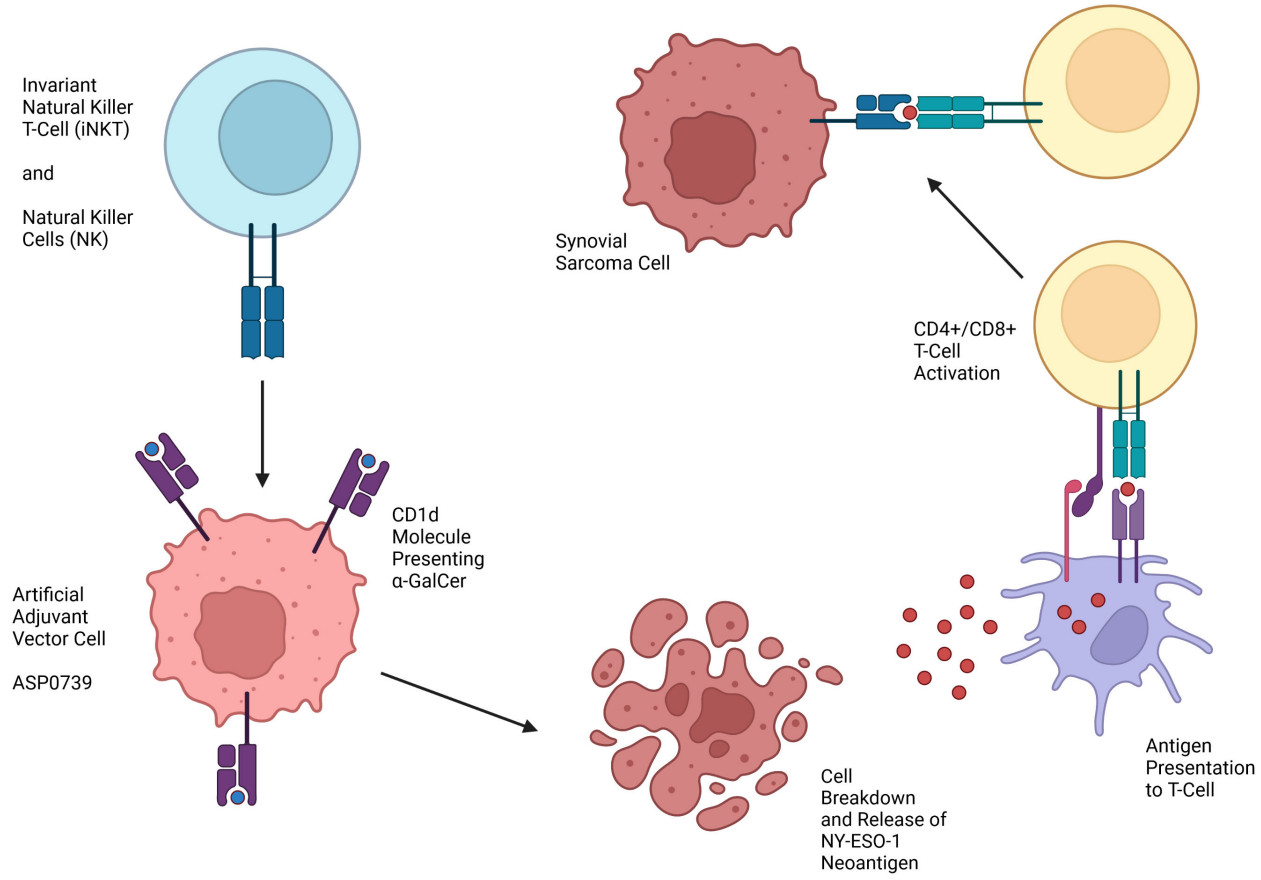

FIGURE 2
Mechanism of action of Artificial Adjuvant Vector Cells targeting NY-ESO-1 for treatment of SYN.

was 52 weeks (8.29 – 75.14). Response rate was higher in patients with fewer lines of previous therapy, smaller target lesions, higher MAGE-A4 scores, those without bridging therapy, women, patients over 40, and patients from North America. The SPEARHEAD-1 trial is currently recruiting for Cohort 2 which will specifically evaluate patients with SYN (36).

2.4 Challenges of cancer testis antigen ACT

There has been success in treating SYN through targeting NY-ESO-1, PRAME, and MAGE-A4 using ACT, with more trial results forthcoming. While this is laudable, there are challenges to the treatment of SYN using these technologies. One barrier is the restriction of many of these therapies to patients with HLA-A*02. Studies have found that HLA-A*02 is more common in Caucasian populations compared to African-American and Asian populations (63). Other barriers for ACT include the multi-week time needed for the production of genetically engineered T-cells, the pre-treatment lymphodepletion regimen which often requires hospitalization, and the high cost of therapy (64, 65). While many of these issues may be overcome through improvement in manufacturing techniques and health systems changes, some may be incontrovertible.

3 NY-ESO-1 artificial adjuvant vector cells

One technology in development for the treatment of SYN that does not require HLA matching is artificial adjuvant vector cells (aAVCs). aAVCs are loaded with an exogenous glycolipid ligand, α-galactosylceramide (α-GalCer), which is presented on a CD1d molecule and activates invariant natural killer T (iNKT) cells (Figure 2) (66). aAVCs also express a specific tumor-associated antigen. The α-GalCer synthetic ligand activating iNKT allows iNKT and natural killer (NK) cells to kill aAVCs, leading to the release of the tumor-associated antigen. Endogenous dendritic cells then serve as antigen presenting cells which allow for creation of CD4+ and CD8+ anti-tumor antigen T-cells. Previously, a Phase II trial of patients with non-small cell lung cancer infused with α-GalCer-pulsed Antigen Presenting Cells (APCs) showed efficacy (67). aAVCs that express NY-ESO-1 have been shown in a murine model to elicit NY-ESO-1 specific CD8+ T-cells as well as have an anti-tumor effect (68).

ASP0739 is an aAVC product targeting NY-ESO-1 being developed for treatment of SYN. Currently, a Phase I trial is in recruitment to test ASP0739 in patients with solid tumors including SYN, myxoid/round cell liposarcoma, ovarian carcinoma, non-small cell lung cancer, and esophageal squamous cell carcinoma (NCT04939701) (43). Phase II of the trial will use ASP0739 in combination with pembrolizumab, an antibody against PD-1 on lymphocytes that prevents de-activation of T-cells by tumors. While the results of these studies are yet to come, these trials will hopefully provide an additional therapeutic opportunity for treatment of SYN without the need for HLA matching.

4 BRD9 targeted therapy

BRD9 small molecule inhibitors are currently in development for the treatment of SYN (Figure 3). Mammalian SWI/SNF (mSWI/SNF or BAF) complexes are chromatin remodelers that allow for alterations in gene expression and DNA transcription. SS18-SSX fusion oncoprotein has been found to hijack the BAF complex, displacing wild-type SS18, resulting in changes in transcription and thus the development of SYN (69). These findings have led to the

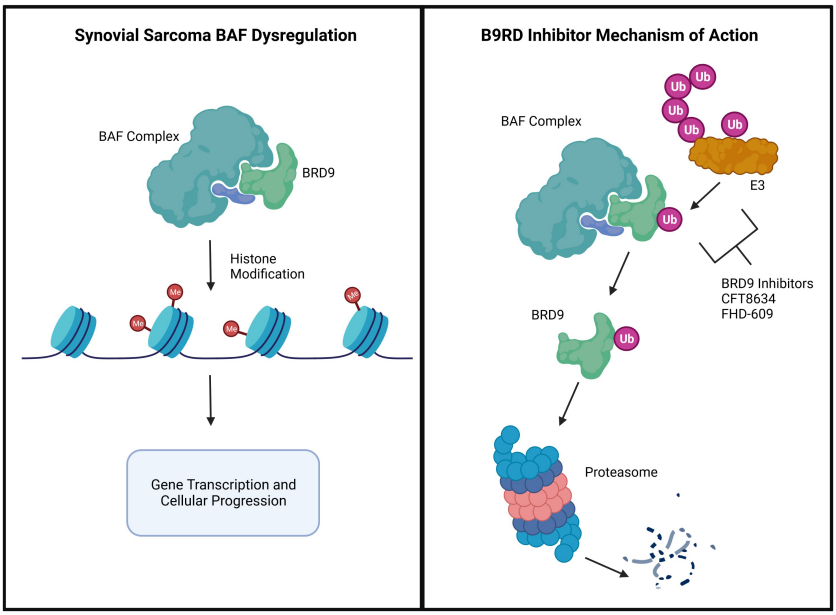

FIGURE 3
BRD9 inhibitor therapy for the treatment of SYN.

recognition of BAF complexes and specific subunits as potential targets for treatment of SYN (70).

BRD9 is a non-BET bromodomain protein and subunit of BAF complexes that has been recognized as a potential target for cancer treatment. In 2017, the first BRD9 chemical degrader was created that bridges the BRD9 bromodomain and E3 ubiquitin ligase complexes *in vitro* (71). Since then, numerous BRD9 inhibitors and have been developed (72–75). Degradation of BRD9 inhibits SYN tumor progression in a murine model (76). Therefore, BRD9 inhibition and/or degradation is a potential target for treatment of SYN.

CFT8634 is an oral heterobifunctional degrader that bridges BRD9 with E3 ligase, causing ubiquitination and proteasomal degradation of BRD9 (77). FHD-609 is an intravenous BRD9 degrader that bridges BRD9 with cereblon (CRBN) E3 ubiquitin ligase substrate that leads to proteasomal degradation (78). These therapies are currently undergoing Phase I trials for patients with advanced SYN (45, 46). The results of these trials are anticipated as potential therapies for treatment of SYN.

5 Conclusion

While standard of care treatment of advanced and metastatic SYN remains anthracycline based chemotherapy, there are numerous technologies in development for the treatment of advanced and metastatic SYN. These technologies stem from improved understanding of the tumor antigen expression and molecular mechanisms behind SYN. Engineered T-cell receptor therapies targeting CTAs has shown success in early-stage trials. Optimization of these engineered TCR treatments is currently being studied, with efforts to enhance T-cell antigen binding, alter the tumor microenvironment, and improve the quality of T-cells used for treatment. Alternative therapies without the need for HLA matching that are currently in recruitment for Phase I trials include aAVCs and BRD9 inhibitors.

Reviewing the new targeted and cellular therapies shows the tremendous progress that has been made over the preceding decades. Nonetheless, further study and qualification are required to ensure that we are doing the best for our patients. We anticipate that with the accelerated pace of discovery and application of new agents, treatment for patients with SYN will make remarkable strides in the upcoming years.

Author contributions

MA conceptualized the manuscript. JRF and JWF wrote the original draft. JRF, JWF, BS, and MA were responsible for writing and editing subsequent drafts and providing final approval for the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MA serves on the advisory board for Aadi Biosciences, Bayer, Deciphera and Regeneron. BS serves on the advisory board for Aadi Biosciences and provides consulting services for Caris Life Sciences.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's note

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Therapeutic advances in leiomyosarcoma

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Leiomyosarcoma is an aggressive mesenchymal malignancy and represents one of the most common subtypes of soft tissue sarcomas. It is characterized by significant disease heterogeneity with variable sites of origin and diverse genomic profiles. As a result, the treatment of advanced leiomyosarcoma is challenging. First-line therapy for metastatic and/or unresectable leiomyosarcoma includes anthracycline or gemcitabine based regimens, which provide a median progression-free survival time of about 5 months and overall survival time between 14-16 months. Effective later-line therapies are limited. Molecular profiling has enhanced our knowledge of the pathophysiology driving leiomyosarcoma, providing potential targets for treatment. In this review, we explore recent advances in our understanding of leiomyosarcoma tumor biology and implications for novel therapeutics. We describe the development of clinical trials based on such findings and discuss available published results. To date, the most promising approaches for advanced leiomyosarcoma include targeting DNA damage repair pathways and aberrant metabolism associated with oncogenesis, as well as novel chemotherapy combinations. This review highlights the recent progress made in the treatment of advanced leiomyosarcoma. Ongoing progress is contingent upon further development of clinical trials based on molecular findings, with careful consideration for clinical trial design, strong academic collaborations, and prospective correlative analyses.

KEYWORDS

sarcoma, soft tissue sarcoma (STS), leiomyosarcoma (LMS), therapeutics, clinical trials

1 Introduction

Leiomyosarcoma (LMS) is a malignant neoplasm of smooth muscle differentiation and is one of the most common subtypes of soft tissue sarcomas (STS) in adults, representing 10-20% of new diagnoses (1, 2). LMS is itself a heterogeneous disease with variable sites of origin, clinical course, and response to therapy, making the treatment of LMS challenging. Common anatomical sites include the uterus, abdomen,

retroperitoneum, and larger blood vessels. LMS of the extremity is less common, accounting for 10-15% of limb sarcomas, with predilection for the thigh (3). Cure may be achieved in patients with localized LMS who undergo surgery, however 40% of cases will still develop local recurrence and/or metastatic disease, most commonly to lung (4). Patients with advanced LMS are typically treated with chemotherapy, either with gemcitabine or doxorubicin based regimens in the first-line setting. Beyond first-line chemotherapy, which provides a median progression-free survival (PFS) of only about 5 months, there are limited treatment options for advanced disease (5).

Molecular profiling has aided in understanding the biology of LMS, providing implications for novel targeted therapies. Based on such profiles, approaches to LMS have evolved and are currently being explored in ongoing clinical trials. In this review article, we describe recent advances in the treatment of advanced LMS. A subset of ongoing clinical trials for patients with LMS is highlighted in Table 1.

2 Tumor biology

The pathophysiology of LMS is complex, making the discovery of effective and targeted treatments challenging. LMS lacks a defining genomic alteration and is instead characterized by substantial mutational heterogeneity with frequent whole-genome duplication, widespread DNA copy-number alterations, and chromothripsis (12–15). The most consistent genomic alterations seen across several studies include mutations or deletions in the tumor suppressors *RB1, PTEN*, and *TP53*. Targetable, activating mutations in oncogenes are rare. Molecular profiling has also uncovered recurrent alterations in telomere maintenance genes such as *ATRX* and homologous recombination DNA repair genes. There has also been evidence for immune infiltration in LMS (16, 17); however, tumor mutational burden is low and microsattelite instability is rare (18, 19).

Due to this genomic heterogeneity, multiomic molecular profiling studies have attempted to further categorize subtypes within LMS. Because LMS may be found in several anatomical sites, investigators asked whether different sites represent molecularly distinct diseases, in particular uterine LMS versus nonuterine/soft-tissue LMS. This is of importance as many clinical trials for LMS are designed to include all anatomical subtypes. From an analysis of 1115 LMS tumors, results suggest that uterine LMS represents a molecularly distinct disease with varying genomic alterations compared with nonuterine LMS (17).

Other studies have identified LMS subtypes that do not necessarily reflect anatomical sites of origin. Molecular subtypes associated with distinct clinical outcomes have been identified by several studies (16, 20–23). Dr. Guo and colleagues demonstrated three reproducible molecular subtypes: Subtype I expressed genes associated with smooth muscle differentiation and demonstrated favorable outcome versus subtype II which expressed less smooth muscle differentiation and had worse prognosis. Subtypes I and II included both uterine and nonuterine LMS whereas subtype III consisted mainly of uterine LMS, which demonstrated intermediate outcome (20). Similarly, Dr. Anderson and colleagues identified three distinct molecular subtypes of LMS that correlate with patient survival. Subtype I (uterine and nonuterine LMS) and subtype III (mainly uterine LMS) harbored a higher overall burden of somatic mutations and were associated with worse survival compared to subtype II (nonuterine LMS of the abdomen/extremity). Furthermore, subtype I was associated with myogenic dedifferentiation and high immune infiltration (16). These data suggest that a subset of uterine LMS behave as an independent molecular subtype while another subset of uterine LMS joins nonuterine LMS to become part of the other identified subtypes.

The identification of varying molecular patterns within LMS highlights the challenges of studying this disease. As of now, patients with LMS are enrolled onto clinical trials as a homogenous entity, ocasionally considering site of disease (uterine versus nonuterine). However, as we have seen, patients with LMS (including those with the same anatomical site) can display vastly different outcomes based on subtype. This can make it difficult to interpret overall results from a trial, as clinically meaningful outcomes may not be directly apparent for certain populations within LMS. Future molecular studies should focus

TABLE 1 Selected available systemic therapies for advanced leiomyosarcoma.

Regimen	Line of therapy	First Author	Phase	Type of Sarcoma	PFS (months)	RR (%)	OS (months)
Doxorubicin v gemcitabine plus docetaxel	First	Seddon (5)	3	STS	5.3 v 5.5	19 v 20	16.3 v 14.5
Trabectedin v dacarbazine Trabectedin v dacarbazine: uLMS subgroup analysis	> 1	Demetri (6) Hensley (7)	3	STS uLMS	4.2 v 1.5 4.0 v 1.5	9.9 v 6.9 11 v 9	12.4 v 12.9 13.4 v 12.9
Pazopanib v placebo Pazopanib: uSTS v non-uSTS subgroup analysis	> 1	Van der Graaf (8) Benson (9)	3	STS STS	4.6 v 1.6 3.0 v 4.5	6 v 0 11.4 v 10.7	12.5 v 10.7 17.5 v 11.1
Eribulin v dacarbazine Eribulin v dacarbazine: LMS subgroup analysis	> 1	Schoffski (10) Blay (11)	3	LMS+LPS LMS	2.6 v 2.6 2.2 v 2.6	4 v 5 5 v 7	13.5 v 11.5 12.7 v 13

LMS, leiomyosarcoma; uLMS, uterine leiomyosarcoma; STS, soft tissue sarcoma; uSTS, uterine soft tissue sarcoma; LPS, liposarcoma; RR, response rate; PFS, progression free survival; OS, overall survival; v, versus.

on identifying actionable targets and biomarkers within these LMS subtypes, which may then be incorporated into future clinical designs and subgroup analyses. Enrollment and treatment selection based on molecular data may ultimately reveal a preferential response for an LMS subtype that would not otherwise be identified.

3 Approved treatments for advanced LMS

3.1 Early line therapies

LMS demonstrates moderate sensitivity to chemotherapy, with uterine LMS being more responsive compared to other anatomical sites (24). In the first-line setting, doxorubicin or gemcitabine based regimens are commonly used. In the phase 2 trial, Gemcitabine and Docetaxel versus Doxorubicin as First-Line Treatment in Previously Untreated Advanced Unresectable or Metastatic Soft-Tissue Sarcoma (GeDDiS), both regimens demonstrated comparable efficacy in STS, including LMS. For gemcitabine and docetaxel versus doxorubicin, there were no significant differences in median progression free survival (PFS) (5.5 versus 5.3 months) or overall survival (OS) (14.5 versus 16.3 months), and objective response rates (ORR) were similar (20% versus 19%). Quality-of-life assessments were compared between the two treatment groups at 12 weeks. There was no significant difference between the two groups at 12 weeks however the mean global health status score was numerically higher in the doxorubicin group versus gemcitabine and docetaxel. This may influence treatment decision for select patients (5).

Although subgroup analysis performed within the GeDDiS trial demonstrated no evidence of differential treatment effect by histologic subtype, other studies in uterine LMS have suggested unique sensitivity to gemcitabine and docetaxel. In a phase 3 study of gemcitabine and docetaxel compared with gemcitabine alone in patients with metastatic soft tissue sarcomas (SARC002), the combination showed superior objective response, PFS and OS. This study also confirmed a higher sensitivity of LMS to gemcitabine and docetaxel compared with other histologic subtypes (25). Subsequently, in a phase 2 study of gemcitabine and docetaxel as first-line treatment for uterine LMS, the ORR was 35.8% with complete response seen in 4.8%, partial response in 31% and stable disease in 26.2% of patients (26). Cross-study comparison is limited however these response findings may imply a more favorable benefit of gemcitabine and docetaxel in uterine LMS versus response data seen in other studies such as GeDDiS. As a result, some prefer gemcitabine and docetaxel as first-line treatment for uterine LMS. Choice of first-line treatment remains individualized, with consideration of many factors including patient preference, performance status, and comorbidities.

Other gemcitabine-based regimens may be considered for early-line treatment of LMS, such as gemcitabine plus vinorelbine and gemcitabine plus dacarbazine. In a phase 2 study of gemcitabine plus vinorelbine in patients with advanced soft tissue sarcomas including LMS who received ≤ 1 prior therapy, clinical benefit (defined as complete response, partial response, or stable disease at > 4 months) was seen in 25% of patients (27). In a randomized phase II study comparing gemcitabine plus dacarbazine versus dacarbazine alone in patients with previously treated STS, median PFS was 4.2 months versus 2 months, median OS was 16.8 months versus 8.2 months, with higher ORR of 49% versus 25% (28). As a result, this regimen may be considered for patients with LMS who failed anthracycline-based treatment.

Early-line therapy for LMS also includes the combination of doxorubicin plus dacarbazine. In a retrospective study of doxorubicin plus dacarbazine, doxorubicin plus ifosfamide or doxorubicin alone as first-line treatment for advanced LMS, 303 patients were included for which 117 (39%) received doxorubicin plus dacarbazine, 71 (23%) received doxorubicin plus ifosfamide, and 115 (38%) received doxorubicin alone. The estimated median PFS was 9.2 months, 8.2 months, 4.8 months, median OS was 36.8 months, 21.9 months, 30.3 months, with ORR of 30.9%, 19.5% and 25.6% for doxorubicin plus dacarbazine, doxorubicin plus ifosfamide, and doxorubicin alone, respectively (29). These data demonstrate favorable activity of doxorubicin plus dacarbazine in LMS and warrant further investigation in prospective clinical trials.

3.2 Later line therapies

Later-line treatment of LMS includes trabectedin, pazopanib, and other chemotherapy agents. Trabectedin is approved in patients with advanced liposarcoma (LPS) or LMS who received prior treatment with anthracyclines. In the randomized phase 3 study of trabectedin versus dacarbazine for metastatic LPS or LMS after failure of conventional chemotherapy, trabectedin demonstrated superior median PFS versus dacarbazine (LMS: 4.3 versus 1.6 months). However, there were no significant differences in OS (12.4 versus 12.9 months) or ORR (9.9 versus 6.9%) (6). In a uterine LMS specific subset analysis of this phase 3 trial, trabectedin provided a median PFS of 4.0 months compared with 1.5 months for dacarbazine, with an ORR of 11% (7). From these data, trabectedin was approved for advanced LMS in October 2015.

Pazopanib is another approved treatment for patients with advanced STS who have previously received chemotherapy, with activity in LMS. Pazopanib is a small-molecule tyrosine kinase inhibitor that inhibits vascular endothelial growth factor (VEGF) receptor, platelet-derived growth factor (PDGF) receptor, and c-KIT (30). In the randomized phase 3 study of pazopanib for metastatic STS (PALETTE), pazopanib demonstrated superior PFS versus placebo (4.6 versus 1.6 months). However, there were no differences in OS (12.5 versus 10.7 months) and objective responses occurred in only 6% of patients (8). In a uterine LMS specific subset analysis, pazopanib provided a median PFS of 3.0 months, OS of 17.5 months, and ORR of 11% (9).

Other chemotherapy agents are also considered for later-line treatment of LMS. Although inferior to trabectedin, dacarbazine demonstrates activity in LMS and is used in the later-line setting (6,

10, 11). In a phase 3 trial of eribulin versus dacarbazine in previously treated patients with advanced LPS or LMS, OS was improved in patients assigned to eribulin. However, an LMS-specific subset analysis demonstrated comparable efficacy for eribulin and dacarbazine (10). Eribulin was approved in January 2016 for LPS, but not for LMS, though the drug is sometimes used for later-line treatment of LMS.

Early-line treatment options for LMS provide a median PFS of approximately 5 months with a median OS of 14-16 months. Later-line regimens are less efficacious, with a median PFS of about 3-4 months, median OS of 12-13 months, with low response rates. Results are summarized in Table 2. There is an urgent need for improved treatment options for patients with LMS. Based on a greater understanding of LMS tumor biology, novel approaches to LMS have evolved and are currently being explored in ongoing clinical trials.

4 Novel approaches to LMS

4.1 Targeting DNA repair pathways

Homologous recombination (HR) comprises a series of interrelated pathways that function in the repair of double-stranded DNA breaks (31). HR deficiency is seen in tumors with loss of *BRCA1/2* function as well-described in ovarian, breast, prostate, and pancreatic cancers. More recently, research has been directed at the concept of "BRCAness" which is a condition in which tumors lack mutations in *BRCA1/2* but harbor alterations in other HR pathway genes resulting in HR deficiency (32). Tumors that display "BRCAness" due to defects in the HR DNA repair pathway may offer opportunities for targeted therapy.

Normally, DNA damage repair is a carefully regulated process in which single-stranded DNA breaks are identified by PARP, resulting

TABLE 2 Selected ongoing clinical trials in leiomyosarcoma.

NCT Identifier	Title	Phase	Line of Therapy	Eligible Subtypes	Status
NCT05633381	Testing Olaparib and Temozolomide Versus the Usual Treatment for Uterine Leiomyosarcoma After Chemotherapy Has Stopped Working	2/3	> 2	uLMS	Recruiting
NCT05116683	ATX-101 in Advanced Dedifferentiated Liposarcoma and Leiomyosarcoma (ATX-101)	2	> 1	LMS	Recruiting
NCT04807816	Targeting ATR in Soft-tissue Sarcomas (TARSARC)	2	0-4	LMS	Recruiting
NCT03536780	Avelumab in Combination With Gemcitabine in Advanced Leiomyosarcoma as a Second-line Treatment (EAGLES)	2	> 1	LMS	Recruiting
NCT04577014	Retifanlimab (Anti-PD-1 Antibody) With Gemcitabine and Docetaxel in Patients With Advanced Soft Tissue Sarcoma	1/2	First	STS including LMS	Recruiting
NCT03138161	SAINT: Trabectedin, Ipilimumab and Nivolumab as First Line Treatment for Advanced Soft Tissue Sarcoma	1/2	>1 (Phase 1); First (Phase 2)	STS including LMS	Recruiting
NCT04551430	Cabozantinib Combined With PD-1 and CTLA-4 Inhibition in Metastatic Soft Tissue Sarcoma	2	2-3	STS including LMS	Recruiting
NCT04624178	A Study of Rucaparib and Nivolumab in People With Leiomyosarcoma	2	2-4	LMS	Not recruiting*
NCT04242238	A Phase 1b Dose Escalation and Dose Expansion Study of a CSF1R Inhibitor (DCC-3014) Administered Concurrently With an Anti-PD-L1 Antibody (Avelumab) in Patients With Advanced High-grade Sarcoma	1b	> 1	STS including LMS	Not recruiting*
NCT03719430	APX005M and Doxorubicin in Advanced Sarcoma	2	Any	STS including LMS	Recruiting
NCT04996004	A Study to Learn About the Study Medicine (Called TTI-621) Given Alone and in Combination With Doxorubicin in People With Leiomyosarcoma (TTI-621-03)	2	Second	LMS	Recruiting
NCT04200443	Cabozantinib and Temozolomide for the Treatment of Unresectable or Metastatic Leiomyosarcoma or Other Soft Tissue Sarcoma	2	0-5	STS including LMS	Recruiting
NCT03016819	Phase III Trial of Anlotinib, Catequentinib in Advanced Alveolar Soft Part Sarcoma, Leiomyosarcoma, Synovial Sarcoma (APROMISS) (APROMISS)	3	> 1	STS including LMS	Not recruiting*
NCT05269355	A Study of Unesbulin in Participants With Advanced Leiomyosarcoma (LMS) (SUNRISELMS)	2/3	> 1	LMS	Recruiting

LMS, leiomyosarcoma; uLMS, uterine leiomyosarcoma; STS, soft tissue sarcoma.
*Not recruiting at the time of publication.

in the recruitment of other DNA damage response proteins. PARP inhibitors (PARPi) result in trapping of PARP at sites of DNA damage, causing replication fork arrest and lethal double-stranded DNA breaks. To resolve this PARP-DNA interaction, HR repair is needed to accurately fix the resulting double-stranded DNA breaks and restart stalled replication forks. In tumors that are HR deficient, double-stranded break repair is imprecise leading to DNA damage accumulation, progressive genomic instability, and cell death (33, 34). Patients with HR deficient tumors may respond more efficaciously to PARPi-based treatment strategies.

LMS, particularly uterine LMS, harbors frequent defects in DNA damage repair based on research from several groups (12, 13, 16, 32, 35–38). In whole-exome and transcriptomic sequencing of 49 LMS patients, deleterious alterations in HR genes were found in the majority of tumors. Enrichment of a mutational signature associated with defective HR repair (Alexandrov-COSMIC mutational signature AC3) was found in at least 57% of cases. In clonogenic assays, LMS cell lines harbored multiple alterations in HR genes and were responsive to the PARPi olaparib in a dose-dependent fashion (13). In a separate cohort of 170 LMS patients from The Ohio State University and the Cancer Genome Atlas, deleterious HR pathway alterations were identified in 23% of patients with uterine LMS and 15% with nonuterine LMS. *BRCA1/2* loss was seen in 10% of the uterine LMS cases and 1% of nonuterine LMS cases. Four uterine LMS patients were treated with off-label olaparib and demonstrated evidence of clinical benefit (35). In another analysis of 211 LMS cases from Memorial Sloan Kettering Cancer Center, deleterious alterations in HR pathway genes were highlighted in uterine LMS compared with nonuterine LMS. About 18% of patients with uterine LMS harbored an HR pathway alteration versus 10% seen in nonuterine LMS (36, 37). Lastly, in a pan-cancer analysis of germline and somatic *BRCA* alterations of several cancers, uterine LMS harbored the highest rate of somatic homozygous *BRCA2* deletion (38).

To investigate the "BRCAness" of LMS and potential for novel targeted therapy, further preclinical evaluations of PARPi have been performed. In the Schwartz laboratory at Columbia University, *in vitro* studies demonstrated limited activity of PARPi monotherapy with olaparib in LMS cell lines. As a result, combination therapies were investigated to potentiate the effects of PARPi. Anti-neoplastic agents such as temozolomide and trabectedin induce DNA damage and are thought to potentiate PARP trapping, leading to increased apoptosis. Additional *in vitro* studies supported this hypothesis in which concurrent treatment with olaparib plus a DNA damaging agent (temozolomide) provided a profound reduction in cell viability of ≥ 90% (32).

PARPi combinations are now being investigated in prospective clinical trials for LMS. The combination of olaparib plus trabectedin was studied in a phase 1b trial by Dr. Grignani and colleagues, where this combination was deemed safe and well-tolerated, with a recommended phase 2 dose (RP2D) of trabectedin at 1.1 mg/m^2 every 3 weeks plus olaparib 150 mg twice a day (39). This led to a phase 2 study of trabectedin in combination with olaparib for advanced unresectable or metastatic sarcoma, which included a cohort of patients with LPS and LMS. Results were presented at the Connective Tissue Oncology Society (CTOS) Annual Meeting in 2022 which demonstrated significant toxicity with this regimen, resulting in frequent dose delays/modifications and discontinuation in 19% of patients overall. For the LMS/LPS cohort, the median PFS was 3.5 months. There were no confirmed objective responses, with best overall response of stable disease in 75% and progressive disease in 25% of patients. As a result, enrollment to stage 2 for the LPS/LMS cohort was not opened (40). A phase 2 study of temozolomide and olaparib for advanced uterine LMS provided encouraging results. In this trial by Dr. Ingham and colleagues, 22 patients who received a median of three prior lines of therapy were treated with temozolomide 75 mg/m^2 once daily in combination with olaparib 200 mg twice daily on days 1-7 of 21-day cycles. The temozolomide plus olaparib combination provided a median PFS of 6.9 months and an ORR of 27%, with a median duration of response of 12 months. Hematologic toxicity was common, as 77% of patients experienced grade 3/4 neutropenia and 32% of patients experienced grade 3/4 thrombocytopenia; however, this toxicity was manageable with dose reduction and there were no events of neutropenic fever or bleeding (41). In correlative analysis, alterations in HR genes including *PALB2* and *RAD51B* or absence of *RAD51* foci formation by a functional assay were observed in patients with prolonged PFS (42). A randomized phase 2/3 trial of olaparib plus temozolomide versus investigator's choice for uterine LMS after chemotherapy failure has initiated recruitment (NCT05633381).

Another potential therapy targeting DNA damage repair pathways in LMS includes the cell-penetrating peptide, ATX-101. Proliferating cell nuclear antigen (PCNA) is a conserved scaffolding protein that interacts with other proteins essential to DNA damage response and intracellular signaling. ATX-101 blocks this interaction and is thought to result in increased cell death through the interruption of DNA damage repair (43, 44). A phase 2 clinical trial investigating ATX-101 monotherapy for advanced LPS and LMS is ongoing (45) (NCT05116683).

Newer approaches to LMS involve directly targeting the cell's main DNA damage response machinery, which is comprised of ataxia telangiectasia and Rad3-related protein (ATR), ataxia telangiectasia mutated (ATM) protein kinase and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (46, 47). As mentioned, molecular profiling has uncovered recurrent alterations in telomere maintenance genes such as *ATRX* and homologous recombination DNA repair genes (13, 16, 17). Mutations in these pathways may lead to increased dependency on the cell's DNA damage response machinery for survival. This has led to appealing anti-cancer targets, including ATR inhibitors (ATRi) and DNA-PK inhibitors (DNA-PKi). The ATRi BAY1895344 was tested *in vivo* in uterine LMS sarcoma mouse models harboring *ATRX* mutations. Treatment with BAY1895344 demonstrated growth inhibition compared to vehicle control, with no significant toxicity (47). Two DNA-PKi were also tested, peposertib and AZD7648 in LMS sarcoma models. Co-treatment with low-dose doxorubicin sensitized LMS cells to peposertib or AZD7648 with significant inhibition of LMS cell viability and proliferation. Furthermore, co-treatment of LMS patient-derived xenografts with peposertib and low dose anthracycline significantly inhibited tumor growth in 5 out of 7 models without toxicity. These responses correlated with HR deficiency and *ATRX* inactivation

(48). Given these promising preclinical data, ATRi are now being studied in patients with sarcoma. A phase 2 study of ATRi berzosertib in combination with gemcitabine for patients with STS is ongoing (NCT04807816).

4.2 Immunotherapy

Immunotherapy has evolved over the past few decades, with tremendous advances in various cancers. Due to these successes, there has been interest in using immunotherapy for the treatment of sarcoma. Several studies have been performed to better understand the tumor immune microenvironment (IME) within sarcoma and translate these findings into novel therapeutic approaches. Dr. Pollack and colleagues investigated the tumor IME in sarcoma for which immunophenotyping of 19 LMS tumors demonstrated a relatively inflamed tumor IME as compared other sarcoma subtypes. For LMS (and undifferentiated pleomorphic sarcoma), there was a higher expression of genes related to antigen presentation and T-cell mediated immunity compared with other subtypes including synovial sarcoma and myxoid/round cell LPS (49). Further investigation within LMS revealed greater immune cell infiltration in soft tissue LMS versus uterine LMS, with soft tissue LMS demonstrating over 2-fold increase in CD8 T-cell and B-cell abundance (50).

Despite the potential for immunotherapy in LMS, clinical trials with immune checkpoint blockade have been disappointing. In the phase 2 trial of pembrolizumab in advanced sarcoma (SARC028), 86 patients were treated, including 10 patients with LMS. There were no objective responses within the LMS population (51). In the phase 2 trial of single agent nivolumab for advanced uterine LMS, none of the 12 treated patients had an objective response and the median PFS was 1.8 months (52). In the phase 2 trials of nivolumab with or without ipilimumab treatment for metastatic sarcoma (Alliance A091401), 43 patients were treated with nivolumab monotherapy, including 15 patients with LMS, and 42 patients were treated with nivolumab plus ipilimumab, including 14 patients with LMS. One of fifteen LMS patients treated with nivolumab monotherapy and two of fourteen LMS patients treated with nivolumab plus ipilimumab demonstrated an objective response, suggesting limited activity in LMS (53).

To potentiate the effects of immune checkpoint blockade, combination approaches with other anti-neoplastic agents such as chemotherapy have been investigated. In a phase 1 trial of gemcitabine and pembrolizumab in LMS and undifferentiated pleomorphic sarcoma, 11/13 treated patients had LMS. There was 1 DLT observed with gemcitabine at 1000mg/m^2. The maximum tolerated dose was not reached and recommended gemcitabine dose was 1200mg/m^2 on day 1 and 8 with pembrolizumab 200mg on day 1, for 21-day cycles. Median PFS was 5.1 months and best response at 9 weeks for LMS was stable disease in 8/11 patients. The final results of the dose expansion cohort are pending (54). In a phase 2 trial of eribulin plus pembrolizumab in patients with metastatic STS, 19 patients with LMS were treated and 11/19 had uterine LMS. The PFS rate at 12 weeks was 42.1% which failed to meet the primary endpoint of 60%. The ORR in the LMS population was 5.3% (55). In a phase 2 trial of pembrolizumab in combination with doxorubicin in patients with anthracycline-naïve advanced STS, 30 patients were enrolled including 10 patients with LMS. The median PFS was 5.7 months for all STS. In the LMS population, 4/10 patients (40%) experienced a partial response, demonstrating encouraging activity of this regimen (56). In a phase 1/2 study of ipilimumab, nivolumab, and trabectedin for advanced soft tissue sarcoma, analysis of phase 2 which included 88 evaluable patients with previously untreated STS demonstrated an ORR of 21.6% with 8 complete responses and 11 partial responses. The median PFS was 7 months and median OS was 14 months (57). In LMS-specific subgroup analysis of this trial which included 19 evaluable patients in phase 2, the ORR was 31.6% with 2 complete responses and 4 partial responses. The median PFS was 7.4 months and median OS was 36.1 months (58). The phase 1 results of a phase 1/2 trial of retifanlimab with gemcitabine plus docetaxel for STS (NCT04577014) were recently presented at ASCO 2022. This study included a safety run-in followed by a 3 + 3 dose de-escalation design. Gemcitabine (900mg/m^2) was administered on days 1 and 8, and docetaxel 75mg/m^2 on day 8, in 21-day cycles. Retifanlimab (210mg IV flat dose in the run-in portion, and 375mg in the dose de-escalation portion) was administered on day 1 of each cycle starting in cycle 2, and continued as monotherapy after 6 cycles of gemcitabine and docetaxel. Results demonstrated safety and tolerability of this regimen, with the RP2D determined to be retifanlimab at 375mg plus gemcitabine and docetaxel. For the run-in and de-escalation cohorts respectively, ORR was 17% and 50%, disease control rates were 100% and 83%, and PFS rates at 24 weeks were 60% and 44%. Phase 2 is ongoing (59). Other active studies testing immune checkpoint blockade in combination with chemotherapy include a phase 2 study of avelumab with gemcitabine, results are pending (NCT03536780).

Other combination approaches with immune checkpoint blockade have been investigated in sarcoma. Synergistic effects have been observed with the combination of immune checkpoint blockade and antiangiogenic agents in other cancers (60). As a result, this approach is of interest in sarcoma. In a phase 2 trial of pembrolizumab with axitinib (a small molecule tyrosine kinase inhibitor active on VEGF receptors) 33 patients were treated including 6 patients with LMS (uterine LMS = 4, non-uterine LMS = 2). Only 1 patient with non-uterine LMS achieved a partial response (61). An ongoing phase 2 trial is testing the combination of cabozantinib (small molecule inhibitor of receptor tyrosine kinases, VEGF, MET, and AXL) with ipilimumab and nivolumab, and is currently enrolling patients (NCT04551430) (62). Another combination approach involves immune checkpoint blockade with PARPi. PARPi may induce DNA damage and enhance the neoantigen burden thereby potentiating the effects of immune checkpoint blockade. This is being studied in a phase 2 trial of rucaparib and nivolumab for LMS (NCT04624178) for which interim results were presented at CTOS 2022. 20 patients were enrolled, for which 75% had uterine LMS, with a median of 2 prior lines of therapy. Based on 17 evaluable patients, median PFS was 7.8 weeks, OS was 9.4 months. There has been 1 partial response in a patient with uterine LMS with a *BRCA2* mutation. 8 (47%) of patients have had a best response of stable disease (63).

LMS is enriched with tumor-associated macrophages compared to other STS subtypes, which may also provide implications for novel targeted therapies. Macrophages are recruited to tumor sites and can interact with neoplastic cells through the release of various growth factors and cytokines, which may promote tumor angiogenesis, invasion, and metastasis. An increased density of tumor-associated macrophages was associated with worse disease-specific survival in LMS (64, 65). It has also been demonstrated that colony-stimulating factor-1 (CSF1) is a major attractant for macrophages expressed by LMS cells. The expression of genes involved in CSF1 signaling was also associated with worse outcomes in both uterine and non-uterine LMS. As a result, strategies have been aimed to deplete tumor-associated macrophages and inhibit CSF1 signaling in sarcoma. In a phase 1b study of avelumab plus DCC-3014 (inhibitor of CSF1 receptor) in patients with advanced sarcoma, 13 patients were treated including 7 patients with LMS. The combination was deemed to be safe and well-tolerated. Study expansion at the recommended phase 2 dose is ongoing (NCT04242238) (66).

Novel immunotherapy agents are also being tested in LMS. CD40 is a master regulator of immunity which mobilizes multiple arms of the immune system to initiate CD8+ T-cell mediated responses against foreign pathogens and tumors. APX005M is a CD40 agonist that is expected to induce an effective anti-tumor immune response in patients with sarcoma (67). A phase 2 trial of APX005M in combination with doxorubicin in STS is actively recruiting patients (NCT03719430). Another targeted approach involves CD47, a widely expressed transmembrane protein which interacts with signal regulatory protein-alpha on the surface of macrophages to protect tumor cells from phagocytosis. CD47 expression is higher in LMS compared with leiomyoma or normal muscle cells (68). In preclinical models of LMS, an anti-CD47 monoclonal antibody demonstrates increased phagocytic activity of LMS cells, thus inhibiting tumor growth and metastatic spread (68). Consequently, a phase 2 trial testing the CD47 inhibitor (TTI-621) is being studied in combination with doxorubicin for patients with LMS (NCT04996004).

4.3 Targeting receptor tyrosine kinases and intracellular signaling pathways

LMS displays substantial mutational heterogeneity and lacks recurrent targetable alterations, including mutations in receptor tyrosine kinases. There are rare circumstances in which actionable gene alterations may be seen in LMS, such as in *ALK*, *FGFR1*, and *NTRK* (17). However, in general due to the lack of targetable mutations, most trials have investigated broadly acting tyrosine kinase inhibitors (TKI) for LMS. As noted earlier, the small-molecule TKI pazopanib is an approved treatment for patients advanced STS who have previously received chemotherapy. However, efficacy in LMS is modest (LMS: ORR = 6%, mPFS = 4.6 months; uterine LMS: ORR 11%, mPFS = 3 months) (8, 9), and there have been ongoing efforts to improve outcomes with other TKIs/TKI combinations.

Clinical trial data examining TKIs in LMS are mixed. Bevacizumab is a monoclonal antibody against VEGF. VEGF normally binds to VEGF receptors, which are family members of receptor tyrosine kinases involved in angiogenesis (69). A phase 3 trial examining the addition of bevacizumab to first-line gemcitabine and docetaxel failed to show improvement in PFS, OS, and ORR (70). Lenvatinib is a small molecule inhibitor that targets fibroblast growth factor receptors (FGFR), PDGFRα, RET, and KIT, in addition to VEGF (71). In a phase 1b/2 study of lenvatinib plus erubulin in advanced LPS and LMS, the phase 1b portion determined the RP2D to be lenvatinib 14mg/day and eribulin 1.1mg/m^2 on day 1 and day 8 for 21-day cycles. A total of 30 patients were enrolled, including 21 patients with LMS. For the LMS population, the median PFS was 8.6 months with ORR of 19% (4/21, 3 uterine and 1 nonuterine LMS) (72). These data may suggest that the addition of lenvatinib potentiates the effects of eribulin, as historical controls of eribulin monotherapy in LMS exhibit worse outcomes, with a median PFS of 2.2 months, OS of 12.7 months, and ORR of 5%, summarized in Table 2 (11). Collectively, these data demonstrate promising efficacy for the treatment of advanced LMS.

Cabozantinib is small molecule inhibitor of tyrosine kinases c-MET and VEGFR2, as well as AXL and RET (73). A phase 2 study testing cabozantinib plus temozolomide for advanced LMS is ongoing (NCT04200443). In a small study performed by Dr. Ikeda and colleagues, the addition of bevacizumab to the regimen of cabozantinib and temozolomide for patients with heavily pre-treated uterine LMS demonstrated improved clinical benefit rate (74). Anlotinib is a multi-target TKI including VEGF1-3, FGFR1-2, PDGFRβ, and KIT (75). A phase 2 trial of anlotinib was tested for first-line treatment in patients with advanced STS, including LMS. Results (all STS) demonstrated a median PFS of 7.1 months, with ORR of 2.7% (76). A randomized phase 3 study of anlotinib versus dacarbazine after failure of prior therapy in several STS subtypes is ongoing, however enrollment in the LMS cohort has been suspended and results are pending (NCT03016819).

Olaratumab is a monoclonal antibody against tyrosine kinase PDGFRα, blocking its interaction with PDGF. A randomized, phase 2 study of doxorubicin plus olaratumab, followed by olaratumab monotherapy in anthracycline-naïve STS demonstrated promising results, with improvement in mPFS and mOS (77). This led to the confirmatory, randomized, phase 3 ANNOUNCE trial of doxorubicin with or without olaratumab in anthracycline-naïve advanced STS, including LMS. For both STS and LMS, there was no significant difference in primary endpoint of mOS between doxorubicin plus olaratumab versus doxorubicin (LMS: 21.6 versus 21.9 months) (78). LMS accounted for a smaller percentage of total subtypes in phase 2 versus phase 3 (36% versus 46.1%), therefore the benefit seen in phase 2 may be weighted towards non-LMS populations (77, 78). Based on these results, olaratumab is not part of standard of care treatments for STS and LMS.

Other approaches to LMS treatment target intracellular pathways involved in tumorigenesis. In LMS, aberrant PI3K/AKT/mTOR signaling has been seen due to *PTEN* loss and amplifications of *IGF1R*, *AKT*, *RICTOR*, and *mTOR* (12). Unfortunately, clinical trials targeting this pathway have demonstrated limited activity. In the phase 2 trial of dual

mTORC1/mTORC2 inhibitor MLN0128 (sapanisertib), 111 patients were treated, including 76 patients with LMS. For the LMS population, PFS was 2.1 months with ORR of 3% (79). Another intracellular target in LMS includes cyclin-dependent kinase inhibition. CDK4 amplification has been seen in some LMS tumors (80, 81). In preclinical models of LMS, treatment with the CDK4/6 inhibitor palbociclib resulted in decreased cell proliferation and induction of G0/G1 phase cell-cycle arrest (81). Consequently, a phase 2 trial of the CDK4/6 inhibitor ribociclib in combination with mTOR inhibitor everolimus was tested in patients with dedifferentiated LPS and LMS with retained Rb expression. 24 patients with LMS were treated, including 14 with uterine LMS. The primary endpoint was progression free rate at 16 weeks, with treatment declared as promising if at least 8/24 patients were progression free at 16 weeks. Final data on the primary endpoint is pending, however of the 22 patients with complete data, 6/22 (27%) met the primary endpoint and median PFS was 19.6 weeks, with no objective responses (82).

4.4 Metabolism

A newer approach to the treatment of sarcoma includes targeting aberrant metabolic processes associated with oncogenesis. In a study of 708 sarcoma tumor samples, argininosuccinate synthase 1 (ASS1) expression was lost in 87%. ASS1 is the rate-limiting enzyme in the conversion of citrulline to arginine in the urea cycle. The loss of ASS1 makes cells dependent on extracellular sources of arginine for survival. As a result, cancer cells lacking ASS1 may have metabolic vulnerabilities (83). Preclinical studies demonstrate synergistic effects with the treatment of arginine depleting enzyme PEGylated arginine deiminase (ADI-PEG20) in combination with gemcitabine and docetaxel. The main transporter of gemcitabine is human equilibrative transporter 1 (hENT1). Priming of tumors with ADI-PEG20 and docetaxel resulted in the stabilization of c-MYC, potentiating the effect of gemcitabine treatment through an increase in hENT1 expression (84).

Given promising preclinical data, a phase 2 study of ADI-PEG20 in combination with gemcitabine and docetaxel for STS was performed by Dr. Van Tine and colleagues. 75 patients who received at least one prior line of therapy were treated. The trial underwent two dose reductions due to prolonged neutropenia and thrombocytopenia: gemcitabine was reduced from 900mg/m^2 to 750mg/m^2, and again to 600mg/m^2. Docetaxel was reduced from 75mg/m^2 to 60mg/m^2. For those receiving gemcitabine 600mg/m^2 + docetaxel 60mg/m^2, PFS and OS were 7.2 and 22.5 months, respectively for the LMS group. 8% of patients (6/75) achieved a complete response, including 3 of the 6 with LMS (85). A phase 3 randomized trial of ADI-PEG20 with gemcitabine plus docetaxel is planned.

4.5 Novel chemotherapy combinations

Currently approved chemotherapy regimens for LMS demonstrate modest efficacy therefore studies have investigated novel chemotherapy combinations in order to improve benefit. Preclinical data demonstrated promising activity with trabectedin and doxorubicin (86, 87). As a result, this combination was studied in two phase 1 studies, which confirmed safety and tolerability when used with granulocyte colony-stimulating factor (88, 89). This led to a phase 3 trial of doxorubicin plus trabectedin versus doxorubicin as first-line treatment for patients with advanced LMS. Patients were randomly assigned (1:1) to receive doxorubicin alone (75 mg/m^2) once every 3 weeks for up to six cycles versus intravenous doxorubicin (60 mg/m^2) plus intravenous trabectedin (1.1 mg/m^2) once every 3 weeks for up to six cycles followed by maintenance with trabectedin alone. The median PFS was significantly longer with doxorubicin plus trabectedin versus doxorubicin alone (12.2 months vs. 6.2 months), at the expense of higher toxicity with grade 3-4 adverse events reported in 52% of patients in the doxorubicin group alone versus 96% in the doxorubicin plus trabectedin group (90).

Another promising novel chemotherapy combination in LMS includes unesbulin (PTC596) plus dacarbazine. Unesbulin is an investigational small-molecule tubulin binding agent. In preclinical LMS models, unesbulin was shown to potentiate the activity of dacarbazine (91). As a result, this was developed into a phase 1b study of unesbulin plus dacarbazine for the treatment of patients with advanced LMS. Results were presented at both ASCO 2022 (92) and CTOS 2022 (93). The RP2D of unesbulin was determined to be 300 mg orally BIW with dacarbazine 1,000 mg/m^2 IV every 21 days. As of the most recent presentation of data at CTOS 2022, there were 33 evaluable patients, 14 with nonuterine LMS and 19 with uterine LMS. Median prior lines of therapy were 3. The ORR was 18.2% with disease control rate of 51.5% at 12 weeks (93). A randomized, placebo-controlled phase 2/3 trial has been developed and is actively recruiting patients (NCT05269355).

5 Discussion

LMS is a rare and aggressive cancer that displays significant clinical and biologic heterogeneity. As a result, LMS is challenging to treat in the advanced setting. Our understanding of LMS pathophysiology has progressed through the use of molecular profiling resulting in the development of novel and targeted treatment approaches. There are several approaches that appear promising thus far. These include targeting DNA damage repair pathways with olaparib and temozolomide, combination chemotherapy with unesbulin plus dacarbazine, several new immunotherapy targets such as CD40 or CSF1 receptor, novel immunotherapy combinations with chemotherapy such as with doxorubicin or with targeted drugs such as cabozantinib, and exploitation of metabolic vulnerabilities using ADI-PEG20 with gemcitabine plus docetaxel. Some of these regimens are now being investigated or will soon be investigated in larger randomized phase 3 clinical trials and have the potential to improve current standards of care in advanced LMS.

The future of LMS treatment is contingent upon a greater understanding of tumor biology and continued development of prospective clinical trials based on molecular findings. A challenge

in studying LMS is how to account for the heterogeneity of this disease, especially in the context of a clinical trial. As we have seen, various multiomic molecular profiling studies have identified subtypes within LMS that display vastly different clinical outcomes and are not necessarily related to anatomical site. Despite this, LMS enrollment onto clinical trials continues as a homogenous entity. There may be clinically meaningful effects of a study drug for certain LMS populations that are not obviously apparent based on overall results, potentially leading to missed therapeutic benefit. This may be the case for several of the negative trials presented in this review, including larger negative phase 3 studies such as ANNOUNCE (78) as mentioned above, EORTC 62012: doxorubicin alone versus combination with ifosfamide (94), PICASSO III: doxorubicin alone versus combination with palifosfamide (95), and TH CR-406/SARC021: doxorubicin alone versus combination with evofosfamide (96). Future enrollment and treatment selection based on molecular data may ultimately reveal a preferential response for an LMS subtype that would not otherwise be identified.

Another challenge in treating LMS is that its most common molecular alterations involve loss of tumor suppressor function in *RB*, *TP53* and *PTEN* (16, 17), which are not currently actionable using existing cancer therapeutics. Furthermore, PD-1 inhibition has not proven efficacious in LMS. New insights into the immunosuppressive features of the LMS tumor IME are needed to identify novel targets for immunotherapy-based approaches. As we have seen, response to immune therapy in LMS is very infrequent and this speaks to the need for biomarker development for this and for other sarcoma subtypes. Tertiary lymphoid infiltrates have been suggested as a biomarker for immunotheapy in sarcomas but this has yet to be fully evaluated prospectively (97).

Future trials should continue to investigate the molecular evolution of LMS, treatment effects on pathology, and discovery of potential biomarkers. Successful translation of molecular findings in LMS will require ongoing preclinical modeling, thoughtful clinical trial design, strong academic collaborations, and prospective correlative analysis. These considerations are necessary for the current and future development of novel therapeutic agents that will improve clinical outcomes for patients with advanced LMS.

Author contributions

KL drafted the manuscript. MI, SB, and GS helped to revise the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MI served as study chair for NCI 10250 phase 2 olaparib and temozolomide.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Targeted therapies for the treatment of soft tissue sarcoma

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Soft tissue sarcomas are rare malignant tumors derived from mesenchymal cells that have a high morbidity and mortality related to frequent occurrence of advanced and metastatic disease. Over the past two decades there have been significant advances in the use of targeted therapies for the treatment of soft tissue sarcoma. The ability to study various cellular markers and pathways related to sarcomagenesis has led to the creation and approval of multiple novel therapies. Herein, we describe the current landscape of targeted medications used in the management of advanced or metastatic soft tissue sarcomas, excluding GIST. We distinguish three categories: targeted therapies that have current US Food and Drug Administration (FDA) approval for treatment of soft tissue sarcoma, non-FDA approved targeted therapies, and medications in development for treatment of patients with soft tissue sarcoma.

KEYWORDS

soft tissue sarcoma, targeted therapy, tyrosine kinase inhibitors, clinical trial, drug therapy

1 Introduction

Soft tissue sarcomas (STS) are rare malignant tumors derived from mesenchymal cells that represent 1% of all adult malignancies in the US (1, 2). In addition to being rare in incidence, treatment of STS is complicated by the heterogenous nature of these tumors. In fact, the 2020 WHO classification of STS includes over 70 different histologic and molecular subtypes which have varied response to treatment (3). The most prevalent soft tissue sarcoma subtypes identified through registries of referral centers other than gastrointestinal stromal tumor (GIST) are liposarcoma, leiomyosarcoma, pleomorphic sarcoma, and synovial sarcoma (4, 5).

First line therapy for most advanced or metastatic STS remains anthracycline-based cytotoxic chemotherapy. For patients with neurotrophic receptor tyrosine kinase (*NTRK*) gene fusion without a known acquired resistance mutation, that are either metastatic or where surgical resection is likely to result in severe morbidity, and who have no satisfactory alternative treatments or whose cancer has progressed following treatment, TRK inhibitors are also a first-line treatment option. Advanced or metastatic STS have high morbidity and

mortality with historic median progression free survival (PFS) of approximately 6 months and median overall survival of just over one year using anthracycline based chemotherapy (6–8) while more recent studies have suggested some improvement in survival with median OS of approximately 20-30 months using anthracycline-based regimens (9, 10).

While some patients with advanced or metastatic disease may benefit from local therapy of oligometastatic disease, for those who have progression on cytotoxic chemotherapy, targeted molecular therapies may be a treatment option. Over the past two decades there have been significant advances in the use of targeted molecular therapies for the treatment of STS. This has altered the landscape of STS therapy and has implications for future targeted therapies for STS which are currently in development.

In this paper we describe the current landscape of targeted therapies that are used in the management of advanced or metastatic soft tissue sarcomas, excluding GIST. We discuss medications in three categories: targeted therapies that have current US Food and Drug Administration (FDA) approval for treatment of STS, non-FDA approved targeted therapies studied in patients with STS, and medications in development for treatment of various STS histologies.

2 FDA approved targeted therapies for treatment of soft tissue sarcoma

2.1 Pazopanib

Pazopanib is an oral small molecule inhibitor of multiple tyrosine kinases including vascular endothelial growth factor receptor (VEGFR)-1, -2, and -3, platelet derived growth factor receptor (PDGFR)-α and -β, stem cell growth factor receptor (c-kit), fibroblast growth factor receptor (FGFR)-1 and -3, and colony-stimulating factor-1 receptor (c-fms) (11). Pazopanib has demonstrated utility in the treatment of all non-adipocytic STS.

In a randomized, double-blind, placebo-controlled phase III trial of 372 patients with non-adipocytic STS who had progression of disease despite standard chemotherapy, pazopanib was found to have a median progression free survival (PFS) of 4.6 months compared with 1.6 months in patients receiving placebo (hazard ratio [HR] 0.31, 95% CI 0.24-.040, $P < 0.0001$) (12). There was no statistically significant improvement in overall survival (OS) with a median OS of 12.5 months with pazopanib group versus 10.7 months with placebo. The most common adverse events (AE) were fatigue (65%), diarrhea (58%), nausea (54%), and weight loss (48%). The most common (≥ 10%) Grade ≥ 3 AE was fatigue (13%). Given the results of this study, pazopanib was FDA approved for the treatment of patients with advanced STS who have received prior chemotherapy in 2012 (13).

Pazopanib has subsequently been studied for patients with specific sarcoma histologies. In a non-comparative, randomized, open-label phase 2 trial of 72 patients with metastatic desmoid tumors, the median PFS for the 43 patients in the pazopanib group was 83.7% (95% CI 69.3 – 93.2) (14). A Phase II study of pazopanib in six patients with metastatic alveolar soft part sarcoma found one patient with partial response and five with stable disease. Median PFS was 5.5 months (95% CI 3.4-7.6 months) and the only severe toxicity noted was one case of Grade 3 diarrhea (15). In a single-arm, phase II trial of 34 patients with metastatic or unresectable typical solitary fibrous tumor, of the 31 evaluable patients, 18 (58%) had partial response 12 (39%) and had stable disease.

While typically used as a subsequent line of therapy after first-line anthracycline, pazopanib has been suggested as an initial treatment option for older adults who may not tolerate anthracycline therapy. An open-label, randomized, phase II study of pazopanib versus doxorubicin has been performed for patients age 60 years or over with progressive advanced or metastatic STS with an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2. This study demonstrated non-inferiority of pazopanib compared with doxorubicin (16).

2.2 Pexidartinib

Tenosynovial giant-cell tumors (TGCTs) are benign neoplasms of joints which, while rarely metastatic, can cause significant morbidity (17, 18). TGCT cells express colony-stimulating factor-1 (CSF1) and frequently have a t(1;2) translocation of the *CSF1* gene on chromosome 1p13 to the *COL6A3* gene on chromosome 2q37 which leads to CSF1 overexpression (19–21). Therefore, CSF1/CSF1R interaction has been considered as a potential therapeutic target in the treatment of TGCT.

Pexidartinib is an orally administered, small molecule tyrosine kinase inhibitor with selective activity against colony stimulating factor 1 receptor (CSF1R) and c-kit (22). Based on its ability to act against CSF1R, pexidartinib was initially studied for the treatment of TGGT in a phase I/II dose-escalation and extension study published in 2015. For the extension group, 12 of 23 patients had partial response and 7 of 23 had stable disease (22).

Given the promising results of this dose-escalation and extension study, a randomized, phase III trial of pexidartinib versus placebo was conducted in 120 patients with advanced TGCT (23). Results of this study showed a 39% overall response rate compared to placebo (0%) ($P < 0.001$). Patients on pexidartinib also reported significantly increased range of motion (+15% with pexidartinib versus +6% with placebo, $P = 0.0043$) and significantly improved physical functioning ($P = 0.0019$) per the Patient-Reported Outcomes Measurement Information System – Physical Function scale (PROMIS). The most common AEs were hair color change (67%), fatigue (54%), aspartate aminotransferase increase (39%), nausea (38%), alanine aminotransferase increase (28%), and dysgeusia (25%). The most common (≥ 10%) Grade ≥ 3 AE were aspartate aminotransferase increase (10%) and alanine aminotransferase increase (10%).

Of note, emergence of mixed or cholestatic hepatotoxicity led to a shortened enrollment period and enrollment was halted six patients short of target. Three patients in the pexidartinib group had aminotransferase levels three or more times the upper limit of normal with total bilirubin and alkaline phosphatase two or more times the upper limit of normal indicative of mixed or cholestatic

hepatoxicity. One patient required two liver dialysis procedures. However, with longer pexidartinib treatment no additional cases of mixed and cholestatic hepatoxicity occurred.

Pexidartinib was FDA approved for the treatment of adult patients with symptomatic TGCT associated with severe morbidity or functional limitations and not amenable to improvement with surgery in August 2019 (24). Given the reports of liver injury, pexidartinib has a boxed warning of hepatoxicity and is available through a Risk Evaluation and Mitigation Strategy (REMS) program.

2.3 Imatinib

Imatinib is an orally bioavailable multikinase inhibitor. Designed as an inhibitor of BCR-ABL, imatinib has been found to have multiple tyrosine kinase activity including against PDGFR-α, -β, and c-kit (25). In 2006 the US FDA approved imatinib for the treatment of adult patients with unresectable, recurrent and/or metastatic dermatofibrosarcoma protuberans (DFSP) which harbors t(17;22)(COL1A1;PDGFB) fusion protein in the majority of cases (26, 27).

Imatinib has been studied in two phase II trials for the treatment of locally advanced or metastatic DFSP harboring t(17;22) and found to have objective response rate approaching 50% (28). The most common (≥10%) Grade 3 AEs of imatinib in the treatment of DFSP are neutropenia (16.7%) and fatigue (16.7%).

Long-term results of a single-institution study of 31 patients with locally advanced/initially inoperable/or metastatic DFSP (including those with fibrosarcomatous transformation) treated with imatinib demonstrated a 5-year PFS of 58% and 5-year OS of 64% (29).

Finally, an updated systematic review published in 2019 showed complete response in 5.2% of patients, partial response rate of 55.2%, and stable disease in 27.6% of 152 patients treated with imatinib for locally advanced or metastatic DFSP (30).

2.4 Crizotinib

Crizotinib is an orally available, small molecule tyrosine kinase inhibitor of c-Met, anaplastic lymphoma kinase (ALK), and ROS1 which has FDA approval for treatment of ALK or ROS1-positive non-small cell lung cancer (31, 32). In January 2022, crizotinib was FDA approved for treatment of pediatric and adult unresectable, recurrent, or refractory ALK-positive inflammatory myofibroblastic tumors (IMT).

Crizotinib has been studied in two open-label trials, one in the pediatric population and one in the adult population. An open-label, phase I dose-escalation study of patients older than 12 months and younger than 22 years with refractory measurable or evaluable solid, CNS tumors, or anaplastic large cell lymphoma was performed (33). Seven patients were enrolled in this study with ALK-positive IMT. Of these patients, 4 had SD and 3 had PR. The most common Grade ≥ 3 AEs was decreased neutrophil count.

In a single-arm, open-label, phase Ib trial of crizotinib for adolescent and adult patients ≥ 15 years old with ALK-positive advanced malignancies other than non-small cell lung cancer, 44 patients were enrolled of which 9 had ALK-positive IMT (34). Of the 9 patients with IMT, 67% (95% CI 30-93) had response with 1 complete response and 5 partial responses. After two years, three of these patients still showed response. The most common Grade ≥ 3 AE for all patients were neutropenia 22.7%, elevated transaminases 6.8%, and vomiting 6.8%.

2.5 Tazemetostat

More than 90% of epithelioid sarcoma (ES) tumors lack expression of INI1/SMARCB1, an epigenetic regulator. Loss of INI1 function allows the histone methyltransferase and epigenetic modifier Enhancer of Zeste Homolog 2 (EZH2) to act as an oncogenic driver in tumor cells (35).

Tazemetostat was developed as an orally available, small molecule selective inhibitor of S-adenosyl methionine (SAM) competitive inhibitor of EZH2 (36). Tazemetostat was initially studied in a phase I trial of relapsed or refractory B-cell non-Hodgkin lymphoma and advanced solid tumors including 3 patients with INI1-negative ES (37).

In an open-label, phase II basket study of patients with INI1-negative solid tumors and synovial sarcoma treated with tazemetostat, results were published for the ES cohort. Of the 62 patients in the ES cohort, tazemetostat showed objective response in 15% of patients at data cutoff and a disease control rate of 26% at 32 weeks (38). The most commonly reported AE were fatigue (37%), nausea (35%), and cancer pain (27%). The only Grade ≥ 3 AE in more than 10% of the study population was anemia (13%). In June 2020, based on the results of this study, the FDA gave accelerated approval for the treatment of patients aged 16 or older with metastatic or locally advanced ES not eligible for complete resection (39).

2.6 Nanoparticle albumin-bound sirolimus (*nab*-sirolimus)

Perivascular epithelioid cell tumor (PEComa) is an ultra-rare type of STS with an estimated annual incidence of ≤1 per 1,000,000 population (40). PEComas often have mutations in or loss of *TSC1* or *TSC2* genes which leads to increased mammalian target of rapamycin (mTOR) activity (41, 42). It is thought that mTOR activation is a driver of cell proliferation in PEComa and mTOR has subsequently been used as a therapeutic target with mTOR inhibitors as evaluated in retrospective analyses and case series (43, 44).

Given the variable oral absorption and bioavailability of sirolimus and everolimus, intravenous nanoparticle albumin-bound (*nab*-sirolimus) has been studied in the treatment of advanced malignant PEComa. Results of a prospective, open-label, phase II registration study of 31 patients who had not previously been treated with mTOR inhibitors and were available

for analysis in the efficacy arm showed an overall response rate of 39% (12 of 31; 95% CI 22 to 58) with one complete response and 11 partial responses (45). Additionally, 52% (16 of 31) of patients had stable disease. Twenty-five patients had tumor profiling. Of note, 8 of 9 (89%) patients with a *TSC2* mutation achieved a confirmed response versus 2 of 16 (13%) without *TSC2* mutation (P <0.001).

The most common AEs ≥ 30% were mucositis (79%), fatigue (59%), rash (56%), anemia (47%), nausea (47%), diarrhea (38%), decreased weight (38%), hyperglycemia (35%), hypertriglyceridemia (32%), hypercholesterolemia (32%), and decreased appetite (32%). The most common (≥10%) Grade 3 AEs were mucositis (18%) and anemia (12%).

When *nab*-sirolimus treatment was expanded in study for use in patients who had been treated previously with mTOR inhibitors (sirolimus, everolimus, temsirolimus, or sapanisertib), 25% (4 of 16 patients) achieved partial response and 50% had stable disease. There were no Grade ≥ 4 AEs (46). *nab*-sirolimus was FDA approved for adult patients with locally advanced unresectable or metastatic malignant PEComa in November 2021 (47).

2.7 Tropomyosin receptor kinase inhibitors

The NTRK genes *NTRK1*, *NTRK2*, and *NTRK3* encode tropomyosin receptor kinase (TRK) proteins known as TRKA, TRKB, and TRKC, respectively (48). While these proteins are normally involved in neuronal development, *NTRK* gene fusions have been identified in a variety of adult and pediatric tumors types (49). These gene fusions encode proteins which have constitutive TRK activity believed to be a key oncogenic driver regardless of tissue type.

Larotrectinib is an orally available, small-molecule inhibitor of all three TRK proteins and has been studied in a phase II basket study of adults and adolescents with *TRK* fusion-positive cancers (50). Seven (13%) patients had infantile fibrosarcoma and 11 (20%) of the patients in the study had "other" soft tissue sarcoma including myopericytoma (two patients), sarcoma that was not otherwise specified (two patients), peripheral-nerve sheath tumor (two patients), spindle-cell tumor (three patients), infantile myofibromatosis (one patient), and inflammatory myofibroblastic tumor of the kidney (one patient).

The overall response rate for all tumor types was 75% (95% CI, 61 - 85) as determined by independent radiology review committee. Of the 55 patients in the study, 7 patients had complete response, 34 had a partial response, and 7 had stable disease. The median time to response was 1.8 months. At 1 year, 71% of responses were ongoing and 55% of all patients remained progression-free.

The most common (>30%) AEs, regardless of attribution, were fatigue (36%), vomiting (33%), nausea (31%), dizziness (31%), and increased ALT or AST (42%). The only Grade ≥ 3 AE regardless of attribution in more than 10% of patients was anemia (11%). Larotrectinib was granted accelerated FDA approval for adult and pediatric patients with solid tumors that have *NTRK* gene fusion without a known acquired resistance mutation, that are either metastatic or where surgical resection is likely to result in severe morbidity, and who have no satisfactory alternative treatments or whose cancer has progressed following treatment in November 2018 (51).

Entrectinib is an orally available inhibitor of all three TRK proteins that has the ability to cross the blood-brain barrier. A review of two phase I (ALKA-372-001 and STARTRK-1) and one phase II (STARTRK-2) clinical trials of entrectinib for NTRK fusion-positive has been conducted (52). There were 13 patients with various types of soft tissue sarcoma included in this analysis.

At the data cutoff (May 31, 2018), the efficacy-available population of 54 adults and 12.9 months of median follow-up showed a 57% objective response including 7% complete response and 50% partial response with a median duration of response of 10 months for all tumor types. The most common (≥10%) Grade ≥ 3 AEs in patients in the NTRK fusion-positive safety population were increased weight (10%) and anemia (12%). Three serious treatment-related events occurred in the NTRK fusion-positive positive population: cognitive disorder, cerebellar ataxia, and dizziness.

In an updated analysis of 150 adults with NTRK fusion-positive tumors treated with entrectinib across 17 solid tumor types, the objective response rate was 61.3% with 16.7% complete responses (53). Thirty-two of the patients in this analysis had NTRK fusion-positive sarcomas and an objective response rate was seen in 19 (59.4%) of these patients. The median duration of response for all NTRK fusion-positive tumor types was 20 months (95% CI 13.2 – 31.1), median progression free survival was 13.8 months (95% CI 10.1 – 20.0), and median overall survival was 37.1 months (95% CI 27.2 – not estimable).

Given that entrectinib crosses the blood brain barrier, patients with CNS metastases were included in this study. In patients with investigator-assessed baseline CNS disease, objective response rate was seen in 61.3% (95% CI 42.2 – 78.2) of patients with baseline CNS metastases compared to 61.3% (95% CI 52.0 – 70.1) in patients without CNS disease.

Entrectinib has been well-tolerated among patients with the most common treatment related AEs being Grade 1/2 including dysgeusia (36.6%), diarrhea (29.8%), and weight increase (28.5%). Adverse events led to dose interruption in 32.8% of patients, dose reduction in 24.3% of patients, and discontinuation in 7.2% of patients.

The most current data on use of entrectinib for NTRK fusion-positive sarcoma was presented at the CTOS Annual Meeting in November 2022 (54). In the sarcoma efficacy population of 26 patients (2 with baseline CNS disease and 24 without baseline CNS disease), 11.5% (2 of 26) had complete response, 46.2% (12 of 26) had partial response, 15.4% (4 of 26) had stable disease. The median duration of response was 15.0 months (95% CI 4.6 – not evaluable). While both patients with baseline CNS disease had at least a partial response, only one patient had a durable response to therapy. Seventeen of thirty-seven patients in the sarcoma safety group experienced a Grade ≥ 3 AE. The most common Grade 3 treatment-related AE was increased weight in 10.8% of patients and there was one Grade 4 treatment-related AE of hyperuricemia.

Entrectinib was granted accelerated FDA approval for adults and pediatric patients 12 years of age and older with solid tumors that that have *NTRK* gene fusion without a known acquired

resistance mutation, that are either metastatic or where surgical resection is likely to result in severe morbidity, and who have no satisfactory alternative treatments or whose cancer has progressed following treatment in August 2019 (55).

3 Non-FDA approved targeted therapies studied in patients with soft tissue sarcomas

3.1 Regorafenib

Regorafenib is a an orally bioavailable multikinase inhibitor of VEGFR-1, -2, and -3, tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2, and KIT (56). It is chemically similar to sorafenib with the addition of a fluorine atom in the center phenyl ring. Regorafenib has met primary endpoints in phase III trials of patients with metastatic colorectal cancer (57, 58), locally advanced, unresectable, or metastatic GIST (59), and hepatocellular carcinoma (60).

In a randomized, placebo-controlled, phase II trial of 182 patients with non-GIST STS subtypes who had progressed or were intolerant to anthracycline-based chemotherapy, compared to placebo, regorafenib was shown to extend PFS in non-adipocytic STS (61). The median PFS for patients with non-adipocytic STS was 4 months with regorafenib vs. 1 month with placebo (HR 0.36, P <0.0001). The most common (≥10%) AEs were asthenia (13%), hand and foot skin reaction (15%), hypertension (18%), and hypophosphatemia (12%).

An open-label, single-arm phase II trial of daily regorafenib for chemotherapy-refractory, metastatic or locally advanced unresectable angiosarcoma demonstrated an overall response rate of 17.4% (4/23) with 52% (12/23) of patients showing progression free survival for greater than 4 months (62). The most common Grade ≥ 3 adverse events were decreased lymphocyte count (26%), hypertension (19%), fatigue (16%), anemia (13%), and hyponatremia (10%). Based on these results, regorafenib has been included as a treatment for metastatic or locally advanced angiosarcoma in the NCCN guidelines (1).

3.2 Sorafenib

Sorafenib is an oral multikinase inhibitor which was initially developed as an inhibitor of Raf kinase. This medication has been found to have broad activity against multiple tyrosine kinases including receptors involved in angiogenesis such as VEGFR-2, -3, and PDGRF-β (63). Given its anti-angiogenic properties, sorafenib has been studied for the treatment of multiple soft-tissue sarcomas including angiosarcoma, desmoid tumor (DT), and solitary fibrous tumor. Sorafenib has been identified as a preferred treatment by the NCCN soft tissue sarcoma guidelines for treatment of DT and solitary fibrous tumor (1).

A double-blind, phase III trial of sorafenib versus matching placebo has been carried out for 87 patients with progressive, symptomatic, or recurrent DTs (64). The primary end point of the study was progression-free survival (PFS). Results of the study showed a two-year PFS of 81% (95% CI 69 – 96) in the sorafenib group and 36% (95% CI 22 – 57) in the placebo group. Results also showed objective response in 33% (95% CI 20 – 48) of the fifty patients in the sorafenib group with one patient having a complete response and 15 having partial responses. Twenty percent (7 of 35 patients) (95% CI 8 – 38) in the placebo group had objective partial response. The most common grades 1 and 2 treatment related adverse events were rash (73%), fatigue (67%), hypertension (55%), diarrhea (51%), and nausea (49%) while the most common Grade ≥ 3 adverse event was rash (14%).

3.3 Imatinib

As previously discussed, imatinib is an oral multikinase inhibitor which has FDA approval for the treatment of locally advanced or metastatic dermatofibrosarcoma protuberans. While not FDA approved for the treatment of locally advanced or metastatic TGCT, imatinib has shown some efficacy for use in this population. A retrospective multi-institutional study of 27 patients evaluable for response showed an overall response rate in 19% of patients with 1 complete response and 4 partial responses and 74% of patients had stable disease (65). It is thought that inhibition of CSF1R by imatinib is the mechanism underlying this response and has led to investigation of CSF1R specific inhibition with medication such as pexidartinib and vimseltinib as discussed.

3.4 Sunitinib

Sunitinib is an orally available tyrosine kinase inhibitor with *in vivo* activity against VEGFR-2 and PDGFR-β (66, 67). Sunitinib has been studied for the treatment of solitary fibrous tumor and alveolar soft part sarcoma (ASPS).

In a retrospective analysis of 31 patients evaluable for response treated with sunitinib for advanced solitary fibrous tumor, the best responses were 2 partial response, 16 stable disease, and 13 progressive disease (67). A <30% decrease in size of tumor was observed in three patients. The median progression-free survival was 6 months.

Sunitinib has also been studied for the treatment of ASPS in a retrospective series of nine patients with advanced, translocated ASPS and evidence of progression during the three months prior to treatment (68). The median progression-free survival was 17 months and there was partial response in 5 cases, stable disease in 3 cases, and progression in one case.

3.5 Lenvatinib

Lenvatinib is an orally administered tyrosine kinase inhibitor that targets VEGFR1-3, FGFR1-4, PDGFRα, c-kit, and RET (69). Lenvatinib has FDA approval for the treatment for the treatment of differentiated thyroid cancer, hepatocellular carcinoma, and as part

of combination therapy in the treatment of renal cell carcinoma and endometrial carcinoma (70, 71)

Pre-clinical evidence has demonstrated activity of lenvatinib in treatment of STS (72). Additionally, phase I dose-escalation studies have shown stable disease using lenvatinib in some patients with synovial sarcoma and leiomyosarcoma (73, 74).

A phase Ib/II study of lenvatinib plus eribulin has been conducted for patients with leiomyosarcoma and LPS (75). Thirty patients enrolled in the study (21 with leiomyosarcoma, 9 with LPS). The objective response rate was 19% for the leiomyosarcoma group and 20% for the LPS group. The median PFS was 8.56 months (95% CI 4.40 – Not Reached) for both groups. The most common Grade ≥ 3 AEs included neutropenia (36.7%), hand-foot syndrome (16.7%), hypertension (13.3%), proteinuria (10%), and febrile neutropenia (10%).

A phase II pilot study evaluating the efficacy of lenvatinib plus pembrolizumab in the treatment of metastatic and/or unresectable soft tissue sarcoma is currently in recruitment (Clinictrial.gov identifier: NCT04784247).

3.6 Crizotinib

In addition to ALK-positive IMT as discussed above, given that ASPS is characterized by translocation between chromosomes 17 and X resulting in *ASPSCR1-TFE3* fusion gene and MET overexpression, crizotinib has been studied in the treatment of advanced or metastatic ASPS (76). A non-randomized, open-label, phase II trial of 45 assessable patients with ASPS was conducted and characterized patients as being MET+ or MET- based on the presence or absence of *TFE3* gene rearrangement (76). Among the 40 MET+ patients, one patient had partial response and 35 had stable disease. The one-year PFS was 37.5% (95% CI 22.9 – 52.1). Among the 4 MET- patients one patient had partial response and 3 had stable disease. The one-year PFS for the MET- group of patients was 50% (95% CI 5.8 – 84.5). One patient had unknown MET status and had stable disease. Grade ≥ 3 treatment related AEs were fatigue in two patients and hypotension with bradycardia, blurred vision, diarrhea, and febrile neutropenia in one patient each, respectively.

3.7 CDK4/6 inhibitors

Palbociclib and abemaciclib are cyclin-dependent kinase CDK4/CDK6 inhibitors which are FDA approved for the treatment of advanced breast cancer (77). Given that a high percentage of well-differentiated (WD) and de-differentiated (DD) liposarcoma (LPS) demonstrate CDK4 amplification, recent trials described below have been conducted to evaluate the utility of CDK4/6 inhibitors in the treatment of LPS.

In a non-randomized, open-label, phase II trial of 60 patients with WD and DD LPS treated with single-agent palbociclib the median PFS was 17.9 weeks (2-sided 95% CI 11.9 - 24.0 weeks) with one complete response. The primary toxicity was neutropenia (grade 3, n = 20 [33%], grade 4, n = 2 [3%]) without neutropenic fever reported (78).

Abemaciclib has also been studied in a single-arm, phase II trial of patients with DD LPS. Thirty patients were enrolled in the study and 29 included for analysis. The median PFS was 30.4 weeks (95% CI 28.9 – NE) with one partial response. The observed PFS at 12 weeks was 76% (95% CI 57-90%). Grade ≥ 3 toxicities included anemia (37%), neutropenia (20%), thrombocytopenia (17%), and diarrhea (7%) (79).

A randomized, double-blind, placebo-controlled phase III study is currently in recruitment for the study of abemaciclib in patients with advanced, recurrent, or metastatic DD LPS (Clinicaltrial.gov identifier: NCT04967521).

While CDK4/6 inhibitors have most evidence for treatment of LPS, a recent phase II study evaluated palbociclib for treatment of other types of STS and osteosarcoma with have high CDK4 expression and underexpressed CDKN2A mRNA (80). Twenty-two patients who had median of three lines of prior treatment were enrolled in the study with nine different sarcoma subtypes, including two osteosarcomas represented. The median follow-up was 10 months, the median PFS was 4.2 months (95% CI 0.9-7.4), and the median 6 months PFS was 30% (95% CI 9-51). Of the 19 evaluable patients, 11 (58%) had stable disease and 8 (42%) had progression as best response. Of note, patients with higher CDK4 expression above the median showed significantly longer median PFS and OS in the univariate analysis.

4 Medications in development for various soft tissue sarcoma histologies

4.1 γ-Secretase inhibitors

The Notch signaling pathway and dysregulation of cross-talk between the Notch and Wnt/β-catenin pathway have been implicated in multiple tumor types including DT (81). γ-secretase inhibitors (GSIs) block Notch receptor proteolysis and subsequent translocation of the Notch intracellular domain to the nucleus, preventing cell cycle progression (82).

The GSI nirogacestat (PF-03084014) was studied in an open-label, phase II trial of 17 heavily pretreated adults with recurrent, progressive DT (83). Results of this study showed a 29% (5 of 17 patients) overall response rate (all partial response) for more than two years. There were also 29% (5 of 17 patients) with stable disease who remained on study. The most common AEs were Grade 1 or 2 (95%) including diarrhea (76%) and skin disorders (71%). The only Grade ≥ 3AE was hypophosphatemia (47%).

Given these results, a randomized, double-blind, placebo-controlled phase III trial of nirogacestat versus placebo has been conducted for patients with progressing DT (84). Results were presented at the European Society of Medical Oncology in 2022. There were 142 patients in the study. Nirogacestat showed improvement in PFS compared with placebo with a HR of 0.29 (95% CI 0.15 – 0.55), overall response rate was 41% with nirogacestat versus 8% with placebo (P<0.001), and the median time to response was 5.6 with nirogacestat versus 11.1 months with placebo. Of the AEs, most were Grade 1 or 2 (95%) and included

diarrhea (84%), nausea (54%), fatigue (51%), hypophosphatemia (42%), and maculopapular rash (32%). Of note, ovarian dysfunction occurred in 75% (27/36) of women of childbearing potential and resolved in 20 (74%) who discontinued the medication.

In addition to the GSI nirogacestat, early studies of the GSIs AL101 and AL102 have demonstrated regression of DT (85, 86). Interim results of a phase II/III open-label dose regimen finding study and randomized, double-blind, placebo-controlled study of AL102 were recently presented at the European Society of Medical Oncology (ESMO) 2022 meeting (ClinicalTrials.gov identifier NCT04871282) (87). As of February 22, 2022, 31 patients had enrolled in the phase II study. Thirty patients were still on study at time of analysis and 18 of those for more than 4 weeks. Mean age was 40 years and 74% of patients were women. The most common treatment-emergent adverse effects (TEAE) ≥ 15% for all doses were diarrhea (39%), rash (26%), nausea (19%), fatigue (19%), and stomatitis (16%). Four patients had Grade 3 AEs (two deemed study-drug related: anemia, diarrhea; two deemed unrelated: vomiting, pleural effusion). There was no significant ECG or food effects noted.

4.2 Anlotinib

Anlotinib is an oral small-molecule inhibitor of multiple tyrosine kinases, primarily VEGFR-2 and -3, FGFR-1-4, PDGFR-α and -β, c-Kit, and Ret (88). Anlotinib first received the National Medical Products Administration of China's approval for use in treatment of locally advanced or metastatic non-small cell lung cancer in 2018 (89). Anlotinib has since been studied extensively in the People's Republic of China and received approval in June 2019 for second-line treatment of clear cell sarcoma, alveolar soft part sarcoma, and other soft tissue sarcomas already treated with first-line anthracyclines (90). This approval was based in part on a phase II study of 166 soft tissue sarcoma patients who had progressive disease after anthracycline-based chemotherapy and had not previously received treatment with angiogenesis inhibitors (91). The results of this study showed twelve-week PFS in 77% of patients with alveolar soft part sarcoma, 75% of patients with synovial sarcoma, and 75% of patients with leiomyosarcoma. The most common grade 3 or higher adverse events were hypertension (4.8%), triglyceride elevation (3.6%), and pneumothorax (2.4%).

Anlotinib (AL3818) is currently being studied in the US as a phase III clinical trial for the treatment of alveolar soft part sarcoma, synovial sarcoma, and leiomyosarcoma. Known as the APROMISS trial, patients with alveolar soft part sarcoma will receive open-label anlotinib while patients with leiomyosarcoma or synovial sarcoma will receive either anlotinib (two-thirds) or dacarbazine (one-third) (Clinicaltrials.gov identifier NCT03016819). At the time of this publication this study is recruiting only patients with alveolar soft part sarcoma.

Preliminary results from the APROMISS trial have evaluated anlotinib compared to dacarbazine for second line treatment of advanced or metastatic synovial sarcoma (92). Seventy-nine patients received initial treatment and were evaluable in this study with 52 receiving anlotinib as the treatment arm and 27 receiving dacarbazine as the placebo arm. Overall PFS was 2.89 months (95% CI 2.73 – 6.87) for anlotinib compared to 1.64 months (95% CI 1.45 – 2.70) for dacarbazine. The primary endpoint was met (P = 0.0015) with a hazard ratio of 0.449 (95% CI 0.270 – 0.744). Grade 3 treatment related adverse events were seen in 23.1% of patients treated with anlotinib and 25.9% of patients treated with dacarbazine. The most common Grade 3 adverse events for anlotinib were diarrhea (5.8%) and hypertension (3.8%).

4.3 MDM2 inhibitors

The *Murine Double Minute Clone 2* (*MDM2*) gene encodes an E3 ligase that binds tumor suppressor P53, both blocking the P53 transactivation domain and targeting P53 for degradation in the proteasome (93). It is thought that inhibition of MDM2 may lead to increased concentrations of P53 and restore P53 function.

MDM2 inhibition is currently being studied in a variety of cancer types given the prevalence of P53 mutations in human cancers. Amplification of MDM2 has been specifically identified in certain cancer types including LPS. In fact, amplification of MDM2 can be useful in the diagnosis of WD LPS (94).

Milademetan, an oral inhibitor of MDM2, was studied in a phase I trial of patients with advanced, relapsed, or refractory solid tumors or lymphoma (95). This study included patients with WD and DD LPS. Fifty percent of the 107 patients in this study had WD/DD LPS. Median age was 61 years and 62% of patients had received ≥3 prior therapies. Partial response was seen in 3.8% of patients and stable disease was seen in 64.2% of patients with WD/DD LPS. The most common (>10%) grade ≥ 3 drug related adverse events in the Schedule D was thrombocytopenia (14%).

Based on these phase I results, milademetan will be studied in a phase III registration study of milademetan compared to trabectedin in patients with unresectable or metastatic DD LPS that has progressed on one or more prior systemic therapies including at least one anthracycline-based therapy (Clinicaltrial.gov identifier: NCT04979442).

In addition to milademetan, BI 907828 is another MDM2-p53 inhibitor currently under study. *In vivo* study of BI 907828 for the treatment of MDM2 amplified DD LPS showed decreased tumor size and even complete response for an *in vivo* murine model (96). Based on these pre-clinical studies, BI 907282 is currently being evaluated in a phase I dose escalation/expansion study of patients with advanced solid tumors (ClinicalTrials.gov Identifier: NCT03449381).

Preliminary results have been presented for a group of 90 patients with median two lines of prior systemic therapy (97). Forty-four of the patients in the study had advanced LPS with 28 diagnosed with DD LPS and 16 diagnosed with WD LPS. At data cut-off, 34.4% of patients had received treatment for ≥ 6 months. In the 41 evaluable patients with LPS, 24 of 27 patients with DD LPS had partial response or stable disease and 13 of 14 patients with WD LPS had partial response or stable disease. The most common Grade ≥ 3 AEs were neutropenia (23.8%), thrombocytopenia (21.4%), and anemia (11.9%).

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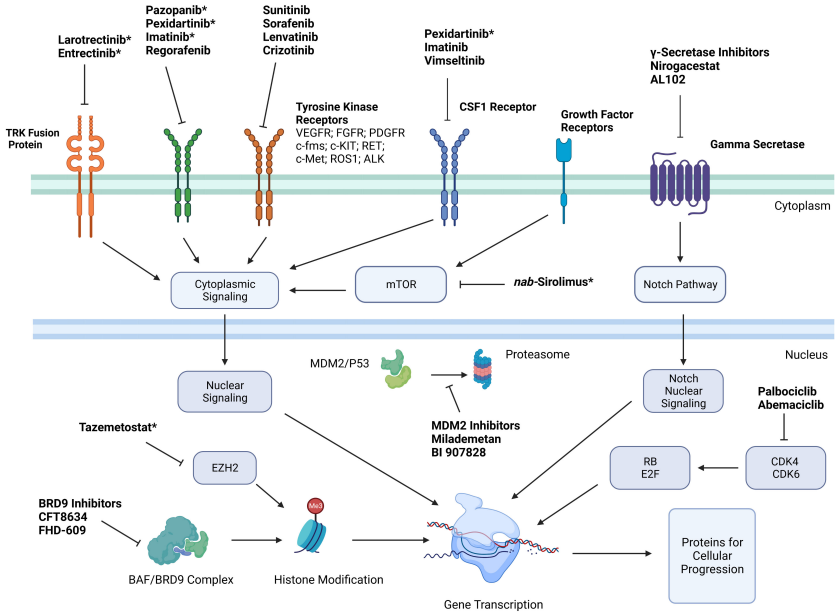

FIGURE 1
Simplified mechanisms of action of targeted therapies for treatment of soft tissue sarcoma. *Indicates that medication is FDA approved for treatment of certain soft tissue sarcoma subtypes. Key: Platelet derived growth factor receptor (PDGFR); vascular endothelial growth factor receptor (VEGFR); stem cell growth factor receptor (c-kit); Hepatocyte Growth Factor Receptor (c-Met); Anaplastic Lymphoma Kinase (ALK); cyclin dependent kinase (CDK); colony stimulating factor 1 (CSF1); enhancer of zeste homolog 2 (EZH2); retinoblastoma (RB).

4.4 Vimseltinib

As discussed above, CSF1/CSF1R interaction has been a recent target for the treatment of TGCT cells given their expression of CSF1 related to the t(1;2) translocation of the *CSF1* gene on chromosome 1p13 to the *COL6A3* gene on chromosome 2q37.

Vimseltinib is an oral, switch control tyrosine kinase inhibitor which has been specifically designed for selective and potent inhibition of CSF1R (98). Initial results from a phase I (dose escalation) and phase II (expansion) study of vimseltinib for treatment of TGCT in patients with unresectable TGCT showed evidence of objective response for 30-50% of patients (99). Updated results from the phase II expansion portion for patients treated with the recommended phase II dose (30 mg twice weekly) showed partial response or stable disease in 100% with 44% of patients in Cohort A and 49% of patients in Cohort B having partial response at a median treatment duration of 7.9 and 5.7 months, respectively (100).

A randomized, double-blind, placebo-controlled, phase III trial is currently in recruitment for study of vimseltinib for patients with unresectable TGCT (Clinicaltrial.gov identifier: NCT05059262).

4.5 BRD9 inhibitors

Synovial sarcoma is defined by the presence of translocation t(X;18)(p11.2;q11.2) leading to the fusion of genes SYT on Chromosome 18 and SSX on Chromosome X (101). The SS18-SSX fusion oncoprotein has been found to result in genetic transcription changes through alteration in the function of SWI/SNF or BAF complexes, leading to the development of synovial sarcoma (102). Changes in canonical BAF (cBAF) complexes driven by the SS18-SSX oncoprotein causes synovial sarcoma gene expression (103, 104). One alteration this leads to is repression of SMARCB1, a cBAF complex protein that may act in tumor suppression and is found in ~70% of synovial sarcoma samples (105).

Studies have found that disruption of the ncBAF complex in samples with loss of SMARCB1 leads to attenuation of cell proliferation in synovial sarcoma (106). One subunit of ncBAF, unique from cBAF and pBAF is the BRD9, a bromodomain-containing protein. Degradation of BRD9 inhibits synovial sarcoma tumor progression in a murine model (107). Therefore, BR9D inhibitors have been developed as a possible target for treatment of synovial sarcoma.

There are two BR9D inhibitors currently under early phase I clinical trial development for the treatment of synovial sarcoma. CFT8634 is an oral heterobifunctional degrader that bridges BRD9 with E3 ligase, causing ubiquitination and proteasomal degradation of BRD9 (108). A phase I clinical trial is currently recruiting to assess the safety and tolerability of CFT8634 in locally advanced or metastatic SMARCB1-Perturbed cancers including synovial sarcoma and SMARCB1-Null tumors who have been previous treated with at least one prior line of systemic therapy (ClinicalTrials.gov Identifier: NCT05355753).

FHD-609 is an intravenous BRD9 degrader that bridges BRD9 with cereblon (CRBN) E3 ubiquitin ligase substrate that leads to proteasomal degradation (109). A phase I, open-label, dose escalation

and expansion study is currently recruiting patients to evaluate the safety, tolerability, and preliminary clinical activity of FHD-609 for patients with advanced synovial sarcoma or advanced SMARCB1-loss tumors (ClinicalTrials.gov Identifier: NCT04965753).

5 Discussion

Targeted therapies for treatment of locally advanced and metastatic STS have historically relied on tyrosine kinase inhibition (TKI) with pazopanib for non-adipocytic STS. Additional TKIs have been studied in STS including imatinib, regorafenib, sorafenib, sunitinib, lenvatinib, and crizotinib. These TKIs are multikinase inhibitors and thought to have activity in treatment of STS given their ability to inhibit angiogenesis and tumor growth promoting receptor tyrosine kinases.

With improved understanding of the cellular markers and possible driver mutations causing sarcomagenesis for different STS subtypes, multiple targeted therapies have been developed to directly inhibit these cellular processes with the hope of objective tumor response. Simplified mechanisms of action of these therapies can be seen in Figure 1. These therapies include FDA approved treatments with a wide variety of specific mechanisms listed in Table 1.

Multiple medications are currently in development for the treatment of STS which are directed at known targets from previously effective therapies including anlotinib (TKI) and vimseltinib (CSF1R inhibitor) and are listed in Table 2. Successive generations of medications targeting known STS drivers may have high receptor affinity and decrease adverse events.

Medications currently under investigation for treatment of STS with novel mechanisms of action include γ-secretase inhibitors (Notch and WNT/β-catenin pathway) for treatment of DT, MDM2 inhibitors targeting P53 for treatment of LPS given high expression of MDM2 in this STS subtype, and BRD9 inhibitors targeting ncBAF complex for treatment of synovial sarcoma and other SMARCB1-loss tumors. These medications and related clinical trials are listed in Table 3.

TABLE 1 FDA Approved Targeted Therapies for Treatment of Soft Tissue Sarcoma.

Medication	Mechanism of Action	Target	Sarcoma Type
Pazopanib	Tyrosine Kinase Inhibitor	VEGFR-1,-2,-3; PDGFR-α,-β; c-kit; FGFR-1,-3; c-fms	Non-adipocytic STS (12)
Pexidartinib	Tyrosine Kinase Inhibitor	CSF1R; c-kit	TGCT (23)
Imatinib	Tyrosine Kinase Inhibitor	PDGFR-β	Dermatofibrosarcoma Protuberans (28–30)
Crizotinib	Tyrosine Kinase Inhibitor	c-Met; ALK; ROS1	IMT (33, 34)
Tazemetostat	EZH2 Inhibitor	EZH2	Epithelioid Sarcoma (38)
nab-Sirolimus	mTOR inhibitor	mTOR Pathway	PEComa (45, 46)
Larotrectinib Entrectinib	TRK inhibitor	TRK	TRK Fusion-Positive Tumors (50, 52–54)

Vascular endothelial growth factor receptor (VEGFR); platelet derived growth factor receptor (PDGFR); stem cell growth factor receptor (c-kit); fibroblast growth factor receptor (FGFR); colony-stimulating factor-1 receptor (c-fms); tenosynovial giant cell tumor (TGCT); hepatocyte growth factor receptor (c-Met); anaplastic lymphoma kinase (ALK); inflammatory myofibroblastic tumor (IMT); mTOR (mammalian target of rapamycin); perivascular epithelioid tumor (PEComa).

TABLE 2 Non-FDA Approved Targeted Therapies Studied in Patients with Soft Tissue Sarcoma.

Medication	Mechanism of Action	Target	Sarcoma Type
Regorafenib	Tyrosine Kinase Inhibitor	PDGFRα; VEGFR-1, -2, -3; c-kit	Non-adipocytic STS (61) Angiosarcoma (62)
Sorafenib	Tyrosine Kinase Inhibitor	Raf Kinase; VEGFR-2, -3; PDGFR-β	Desmoid Tumor (64)
Imatinib	Tyrosine Kinase Inhibitor	ABL; PDGFR; c-kit Possible CS1FR	TGCT (65)
Sunitinib	Tyrosine Kinase Inhibitor	VEGFR-2, PDGFR-β	Solitary Fibrous Tumor (67) ASPS (68)
Lenvatinib	Tyrosine Kinase Inhibitor	VEGFR1-3; FGFR1-4; PDGFRα; c-kit; RET	Leiomyosarcoma (75) LPS (75)
Crizotinib	Tyrosine Kinase Inhibitor	c-Met; ALK; ROS1	ASPS (76)
Palbociclib Abemaciclib	CDK4/6 Inhibitor	CDK 4/6	WD/DD LPS (78, 79) STS with high CDK4 expression (80)

Platelet derived growth factor receptor (PDGFR); vascular endothelial growth factor receptor (VEGFR); stem cell growth factor receptor (c-kit); hepatocyte growth factor receptor (c-Met); anaplastic lymphoma kinase (ALK); cyclin dependent kinase (CDK); tenosynovial giant cell tumor (TGCT); Alveolar Soft Part Sarcoma (ASPS); well-differentiated/dedifferentiated liposarcoma (WD/DD LPS).

TABLE 3 Medications in Development for Treatment of Various Soft Tissue Sarcoma Histologies.

Medication	Mechanism of Action	Target	Sarcoma Type	ClinicalTrials.gov Identifier
Nirogacestat AL102	γ-Secretase Inhibitors	Notch and Wnt/β-catenin pathway	Desmoid Tumor	NCT03785964 NCT04871282
Anlotinib	Tyrosine Kinase Inhibitor	VEGFR-2,-3; FGFR-1,-4; PDGFR-α,-β; c-Kit; Ret	ASPS Synovial Sarcoma Leiomyosarcoma	NCT03016819
Milademetan BI 907828	MDM2 Inhibitor	P53	WD/DD LPS	NCT04979442 NCT03449381
Vimseltinib	Tyrosine Kinase Inhibitor	CSF1R	TGCT	NCT05059262
CFT8634 FHD-609	BRD9 Inhibitor	ncBAF Complex	Synovial Sarcoma SMARCB1-Loss Tumors	NCT05355753 NCT04965753

Vascular endothelial growth factor receptor (VEGFR); platelet derived growth factor receptor (PDGFR); fibroblast growth factor receptor (FGFR); alveolar Soft Part Sarcoma (ASPS); well-differentiated/dedifferentiated liposarcoma (WD/DD LPS); Murine Double Minute Clone 2 (MDM2); tenosynovial Giant Cell Tumor (TGCT).

Given the interest in immunotherapy for treatment of STS, future studies may seek to combine targeted therapy with immunotherapy to evaluate if there is enhancement in treatment effect and improved patient outcomes (110, 111). These studies must be mindful of adverse effects of combination immunotherapy as has been seen in previous study (112, 113).

In addition to therapies that target specific cellular and molecular mechanisms as discussed, research is also underway to identify drug delivery systems which may improve patient outcomes. Nanoparticle albumin-bound sirolimus (*nab*-sirolimus) is an example of a targeted therapy (mTOR inhibitor) which had improved therapeutic dosing with a nanoparticle drug delivery system. Future work will explore drug delivery systems with the hope to enhance the effect of chemotherapy, molecular targeted therapies, and radiation therapy while reducing toxicity (114).

6 Conclusion

Over the past two decades there has been significant advancement in the use of targeted therapies for the treatment of advanced and metastatic STS. These developments in targeted therapies have highlighted a key paradigm and future direction of treatment. Continuing in this vein, and building on the success of the prior years, it is easy to see that the future of treatment in sarcoma is bright. Next generation sequencing of STS in later lines will continue to improve, and with it, our ability to identify actionable targets. The promise of treatments that minimize toxicity, while maximizing on target efficacy is hard to ignore, and with the rapid pace of development, may shortly be in reach.

Author contributions

MA conceptualized the manuscript. JWF and JRF wrote the original draft. JRF, JWF, BS, and MA were responsible for writing and editing subsequent drafts and providing final approval for the manuscript. All authors contributed to the article and approved the submitted version.

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Figure created in Biorender.com.

Conflict of interest

MA serves on the advisory board for Aadi Biosciences, Bayer, Deciphera and Regeneron. BS serves on the advisory board for Aadi Biosciences and provides consulting services for Caris Life Sciences.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's note

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New therapeutics for soft tissue sarcomas: Overview of current immunotherapy and future directions of soft tissue sarcomas

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Soft tissue sarcoma is a rare and aggressive disease with a 40 to 50% metastasis rate. The limited efficacy of traditional approaches with surgery, radiation, and chemotherapy has prompted research in novel immunotherapy for soft tissue sarcoma. Immune checkpoint inhibitors such as anti-CTLA-4 and PD-1 therapies in STS have demonstrated histologic-specific responses. Some combinations of immunotherapy with chemotherapy, TKI, and radiation were effective. STS is considered a 'cold', non-inflamed tumor. Adoptive cell therapies are actively investigated in STS to enhance immune response. Genetically modified T-cell receptor therapy targeting cancer testis antigens such as NY-ESO-1 and MAGE-A4 demonstrated durable responses, especially in synovial sarcoma. Two early HER2-CAR T-cell trials have achieved stable disease in some patients. In the future, CAR-T cell therapies will find more specific targets in STS with a reliable response. Early recognition of T-cell induced cytokine release syndrome is crucial, which can be alleviated by immunosuppression such as steroids. Further understanding of the immune subtypes and biomarkers will promote the advancement of soft tissue sarcoma treatment.

KEYWORDS

soft tissue sarcoma, immune checkpoint inhibitor, adoptive immunotherapy, cancer testis antigen, T-cell receptor therapy, chimeric antigen receptor (CAR) T-cell, tumor-infiltrating lymphocyte, tumor microenvironment

1 Introduction

Sarcomas are a rare and heterogeneous group of solid tumors of mesenchymal origin, accounting for only 1% of all adult malignancies. They can be divided broadly into soft tissue sarcomas (STS), which originate in the fat, muscle, nerve, nerve sheath, blood vessels, and other connective tissues or the bone.

More than 70 different histologic subtypes of STS have been identified (1). Soft tissue sarcoma is an aggressive disease with a 40 to 50% metastasis rate, with a 5-year survival rate of 30%. STS most commonly metastasizes to the lungs; tumors in the abdominal cavity more commonly metastasize to the liver and peritoneum (2).

The limited durable response with traditional surgery, radiation, and chemotherapy in advanced-stage sarcoma has prompted research in novel immunotherapy of soft tissue sarcoma.

1.1 Immune microenvironment of sarcoma

The tumor microenvironment (TME) comprises a tumor, stromal cells, and immune cells such as macrophages, lymphocytes, and extracellular matrix (3). Tumor cells take advantage of TME over time, and genetic/epigenetic changes of the tumor and rearrangement of TME are pivotal in tumorigenesis (4).

Tumor associated macrophages (TAMs) are distinguished components in TME. Tumors secrete high levels of colony-stimulating factor 1 (CSF-1), which converts M1 macrophage (classically activated, tumoricidal) to M2 macrophage/TAMs (alternatively activated, tumor-promoting) and stimulates tumor growth and metastasis along with CCL2 (5).

Sarcoma is traditionally considered an immunologically quiet tumor with low tumor mutational burden (1.06 mutations/Mb) and immunosuppressive TME (high levels of hypoxia-inducible factor 1 α (HIF1α), macrophages, neutrophils, and decreased T-cell levels) (6). A subset of sarcomas are sensitive to ICIs. They are 'hot'/immune-sensitive tumors with high TMB, interferon, CD8 lymphocytes, and PD-L1 expression (7, 8).

A very recent paper highlights the significant prognostic value of systemic inflammatory indexes as a prognostic marker in terms of PFS and OS in STS patients who progressed on anthracycline. A low lymphocyte-to-monocyte ratio (LMR) was associated with worse OS ($p = 0.006$). Interestingly, low lymphocyte-to-monocyte ratio (LMR) was an indicator of trabectedin efficacy, which could be applied in clinical practice (9). In a previous study in 2021, 3D-cultured cells from leiomyosarcoma and undifferentiated pleomorphic sarcoma (UPS) surgical specimens were treated with trabectedin and demonstrated the involvement of ECM-associated genes such as *mmps* and their inhibitor *timp1*, emphasizing the potential role of ECM in the activity of trabectedin (10).

It was proposed that tumors with high PD-1 expression and tumor-infiltrating lymphocytes (TILs) respond well to ICIs (11). Sarcomas have relatively low PD-1 and TILs. Various studies have revealed conflicting results regarding how PD1 expression impacts prognosis. A recent review of Phase II trials demonstrated that 30% of patients with PD-L1 expression (≥1%) achieved a response. However, 7% of PD-L1 negative patients also achieved a response, underscoring the limitation of PD-L1 as a prognostic marker (12). A subsequent analysis of SARC028 revealed that higher TILs at baseline were associated with a better PFS.

In this article, we will review current immunotherapy of soft tissue sarcoma, highlighting prominent trials with immune checkpoint inhibitors and adoptive cellular therapies, including engineered T-cell receptor targeting cancer testis antigens (CTA), chimeric antigen receptor (CAR) T-cell therapies and tumor-infiltrating lymphocytes (TILs).

2 Immune checkpoint inhibitors

Immune checkpoint inhibitors (ICI) regulate critical inhibitory signals of T-cells such as PD-1/PD-L1 and CTLA-4 axes as monotherapy or in combination with chemotherapy. ICIs are FDA-approved to treat more than 50 cancer types, including advanced solid tumors, MMR-deficient tumors, and tumors with a high tumor mutation burden (13).

SARC028 was a significant Phase II trial published in 2017, which first demonstrated the efficacy of pembrolizumab (PD-1 inhibitor) in some STS, notably in undifferentiated pleomorphic sarcoma (UPS) (4 of 10) and dedifferentiated liposarcoma (dLPS) (2 of 10) (14). The final results of SARC028 expansion cohorts confirmed effectiveness in UPS, with an objective response rate (ORR) of 23%, but not in dedifferentiated/pleomorphic liposarcoma (LPS) with an ORR of 10% (15).

In the Phase II Alliance A091401 trial, patients with metastatic sarcoma were treated with nivolumab (PD-1 inhibitor) with or without ipilimumab (CTLA-4 inhibitor). Dual immune checkpoint blockade demonstrated an overall response (ORR) of 16%. Responses were confirmed in leiomyosarcoma (uterine (n=1), non-uterine (n=1)), myxofibrosarcoma (n=1), UPS (n=2), and angiosarcoma (n=1) (16). In a phase II study for advanced uterine leiomyosarcoma, none of the 12 patients responded to nivolumab alone (17). In a subsequent Phase II expansion cohort study, combination therapy of nivolumab and ipilimumab resulted in an ORR of 28.6% in UPS and 14.3% in dedifferentiated liposarcoma (18). In a DART trial by SWOG, a phase II trial of ipilimumab and nivolumab in angiosarcoma demonstrated an ORR of 25% (19). On December 2022, atezolizumab was granted FDA approval for unresectable or metastatic alveolar soft part sarcoma (ASPS) (ORR = 24%, NCT03141684).

Myxofibrosarcoma (MFS) expresses high levels of immune microenvironment markers, and some case reports support PD-1 inhibition in myxofibrosarcoma, which is further explored in a Phase II trial (ENVASARC, NCT04480502) (20–23).

ICI response in soft tissue sarcoma has been modest and histologic-specific, especially in UPS, dLPS, ASPS, and angiosarcoma.

2.1 ICI and local/systemic therapy

Combinational strategies with ICI and local/systemic therapies can overcome soft tissue sarcoma resistance mechanisms. Local therapies to complement ICI consist of isolated limb infusion and radiation.

Isolated limb infusion (ILI) is a minimally invasive administration of high-dose chemotherapy to treat STS in the extremities (24). Two patients with recurrent myxofibrosarcoma responded to melphalan *via* ILI and pembrolizumab (1=partial response, 1=complete response) (25). This promising case

prompted a subsequent Phase II trial with pembrolizumab plus the infusion of melphalan and dactinomycin (NCT04332874).

Radiation therapy is another local therapy to activate anti-tumor immunogenicity in the tumor microenvironment through the cGAS-STING pathway and subsequent CD8+ T cell activation (26, 27). There are approximately ten ongoing trials to investigate the effect of radiation in addition to ICI.

Chemotherapy enhances immunosurveillance by releasing type I interferon (IFN), and increasing M2 macrophages, CD8+ T cells, and NK cells in a tumor microenvironment (28, 29).

Two Phase II trials of doxorubicin and pembrolizumab from Pollack et al. and Livingston et al. demonstrated promising ORR of 19% in advanced sarcoma and 36.7% in advanced STS, respectively (30, 31). In a Pollack et al. study, grade 3+ treatment-related adverse effects (TRAEs) such as neutropenia (6/37), leukopenia (1/37), and febrile neutropenia (1/37), heart failure due to doxorubicin (2/37), and adrenal insufficiency (1/37) and hypothyroidism (7/37) due to pembrolizumab were observed. In a Livingston et al. study, grade 3+ TRAEs include neutropenia and leukopenia (11/30 each), and anemia (8/30). Arthralgia (3/30), fatigue (2/30), autoimmune disorder (2/30), and increased lipase (2/30) were grade 3+ TRAEs attributed to pembrolizumab. Additionally, pembrolizumab-related synovitis/myositis (n=1), autoimmune hepatitis (n=1), and autoimmune nephritis (n=1) were observed, and all patients responded to steroids. Grade 5 adverse events were not reported in both studies.

Trabectedin, in addition to ipilimumab and nivolumab, revealed an ORR of 19.5% in metastatic STS (32). Grade 4 adverse events include anemia, neutropenia, thrombocytopenia, and increased AST/ALT and CPK. Grade 5 rhabdomyolysis was observed in one patient.

Another strategy to augment immune response in STS is to combine small molecule inhibitors such as tyrosine kinase inhibitors (TKI). In the Phase II Immunosarc trial, TKI sunitinib with nivolumab in metastatic or locally advanced STS led to an ORR of 21%, with 48% of 6-month PFS (33). Wilky et al. demonstrated the efficacy of Axitinib (VEGF receptor TKI) and pembrolizumab in advanced sarcoma. None achieved a complete response. 8 out of 32 patients achieved a partial response (ORR 25.0%), with most responses occurring in ASPS (6/11, ORR 54.5%) (34).

Pembrolizumab is FDA-approved in many cancers such as advanced melanoma, Merkel Cell Carcinoma, Cutaneous Squamous Cell Carcinoma, and non-small cell lung cancer, either alone or with other therapies (35–38).

Phase II trials combining systemic therapy with pembrolizumab in sarcoma are in progress: Pembrolizumab + eribulin (NCT03899805), pembrolizumab + gemcitabine (NCT03123276), pembrolizumab + lenvatinib (NCT04784247), pembrolizumab + doxorubicin (NCT03056001), pembroliumab + cabozantinib (PEMBROCABOSARC, NCT05182164), pembrolizumab + epacadostat (IDO1 Inhibitor)(NCT03414229).

Other PD-1 inhibitors in sarcoma are investigated in Phase II trials. Nivolumab + Gemcitabine/Doxorubicin/Docetaxel (GALLANT, NCT04535713), Retifanlimab (PD-1 inhibitor) + Gemcitabine/Docetaxel (NCT04577014), Sintilimab (PD-1 inhibitor) + Doxorubicin/Ifosfamide (NCT04356872) and Camrelizumab (PD-1 inhibitor) + Doxorubicin/Ifosfamide (NCT04606108) are in progress.

Future research should aim to identify biomarkers in STS to augment responses of ICI with and without local/systemic therapies in each patient.

3 Adoptive cellular therapies

Successful T-cell treatments for hematological malignancies have sparked interest in researching T-cell therapies for solid tumors such as sarcomas.

One of sarcoma's primary immune evasion strategies is inadequate neoantigens/antigen recognition, which fails to create enough tumor-specific T cells and immune responses. Adoptive cellular therapies hope to avoid this phase by supplying a significant amount of autologous T cells specifically designed for a particular antigen. Autologous T cells are obtained from peripheral blood or the original tumor and then amplified. Potential approaches include engineered T-cell receptor (TCR) and chimeric antigen receptor (CAR) T-cell therapy and tumor-infiltrating lymphocyte (TIL) therapy with sarcoma.

3.1 Engineered T-cell receptor therapy

Cancer testis antigens (CTA) are tumor-associated antigens (TAA) that are typically present in fetal development (placenta and embryo) or at immune-privileged sites without MHC class I (testes) (39). Sarcomas express higher than normal CTAs, especially in SS and myxoid/round cell liposarcoma (40, 41). Sarcomas express a variety of CTAs such as the NY-ESO-1, MAGE, and GAGE family and fetal acetylcholine receptors (42).

NY-ESO-1 and MAGE family are intracellular antigens that must be processed and presented with MHC. TCR T cells require patients with matching HLA allele subtypes, often HLA-A2, which compose approximately 30% of the population. Modified TCR T cells recognize processed peptides *via* HLA-A2-specific manner and mount immune responses (43).

In 2011, Robbins et al. successfully investigated the antitumor response of NY-ESO-1-specific TCRs with high dose interleukin-2 in refractory synovial sarcoma (SS). Objective clinical responses were observed in 4 of 6 SS patients. A partial response lasted for 18 months in a patient with synovial sarcoma (44). Long-term follow-up study which enrolled 12 additional SS patients, revealed that 11 of 18 patients with SS who received anti-NY-ESO-1 TCRs responded to therapy (61%), and one had a complete response (45).

In a Phase I trial in 2018, T cells expressing NY-ESO-1c259 (Letetresgene autoleucel), a modified TCR recognizing NY-ESO-1/LAGE1a peptide, demonstrated an ORR of 50% (6/12) in metastatic SS following a lymphodepleting regimen of fludarabine and cyclophosphamide. Remarkably, self-generating pools of NY-ESO-1c259T cells persisted *in vivo* for at least 6 months in all patients who responded. No fatal adverse events were reported. Grade 3-4 adverse events include lymphopenia, leukopenia, neutropenia, anemia, thrombocytopenia, and hypophosphatemia.

Cytokine release syndrome was reported in five patients, with median onset within 4 days and a median duration of 10 days (46).

High dose fludarabine-containing regimen is necessary for the efficacy of NY-ESO-1c259 TCR, likely correlated with elevated IL-7 and IL-15, and TAM modulation (47).

Afamitresgene autoleucel (ADP-A2M4 SPEAR TCRs directed against the MAGE-A4) revealed comparable efficacy. Phase I study with MAGE-A4c1032 TCR by Hong et al. observed an ORR of 25% in advanced solid tumors, and all partial responses were in patients with synovial sarcoma. Two patients had trial-related deaths due to aplastic anemia and CVA (48). A subsequent phase II study with afamitresgene autoleucel revealed an ORR of 40% in 25 patients with a tolerable safety profile in advanced/metastatic SS and Myxoid/Round Cell Liposarcoma (MRCLS) (49).

Although engineered TCR in advanced soft tissue sarcoma presents promising efficacy, there are some limitations to overcome, particularly the HLA-A2 requirement, manufacturing timelines/cost, and associated toxicities such as cytokine release syndrome. Furthermore, there are heterogenous CTA expressions in different types of sarcomas, and broad applicability may be limited (43).

3.2 Chimeric antigen receptor T-cell therapies

CARs are chimeric antigen receptors artificially engineered to recognize naturally occurring tumor surface antigens and activate T-cells in an MHC-independent manner (50).

C19-targeted CAR T-cell therapies for hematologic malignancies such as CD19-positive B-cell acute lymphoblastic leukemia and B-cell lymphomas have been successful. In 2022, Ciltacabtagene autoleucel, B-cell maturation antigen-directed CAR T-cell, was FDA-approved for patients with refractory or relapsed multiple myeloma who received at least four lines of therapy (CARTITUDE-1, NCT03548207). Further efforts to expand CAR T-cell therapies in solid tumors are ongoing but have not shown major significance yet.

In Phase I/II trial in HER2-positive sarcomas, including 16 osteosarcomas, one Ewing sarcoma, one primitive neuroectodermal tumor, and one desmoplastic small round cell tumor, HER2-CAR T cell therapy induced stable disease in four patients without significant toxicity (51).

In another Phase I trial, ten HER2+ refractory/metastatic patients (osteosarcoma (5), rhabdomyosarcoma (3), Ewing sarcoma (1), and synovial sarcoma (1)) were enrolled and treated with HER2-CAR T cells and lymphodepletion with either fludarabine or in combination with cyclophosphamide. At the initial follow-up at 6 weeks, 4 patients had progression, and 4 patients achieved stable disease. Overall survival at 1 year was 60% for patients treated with HER2-CAR T cells and lymphodepletion (52).

EGFR, GD2, insulin-like growth factor 1 receptor (IGF-1R), tyrosine kinase orphan-like receptor 1 (ROR1), CD44v6, and NK cell activating receptor group 2-member D (NKG2D) are potential targets in sarcoma, and early phase trials are underway to investigate the efficacy of CAR therapies for these targets.

CAR T-cell therapies will have to overcome a few obstacles in the future. CAR T-cell therapies have limited cancer-specific antigens, whereas TCRs recognize peptides presented *via* MHC class 1, which essentially include whole proteasome (53, 54). Until now, CAR-T therapies seek more specific targets in solid tumors, which are conserved and do not convey toxicity to healthy tissue, to improve long-term efficacy (55).

Cytokine release syndrome (CRS) is one of the adverse effects of both TCR and CAR T-cell therapy following T-cell administration. CRS is an acute, systemic response from immune stimulation in an "on-target and on-tumor" manner. T-cell therapies can also induce unexpected "on-target, off-tumor" autoimmunity, which damages healthy cells by recognizing shared antigens (56–58). It is crucial to promptly recognize and treat immune-mediated adverse effects, which can be alleviated by immunosuppression such as Tocilizumab and steroids if needed.

3.3 Tumor-infiltrating lymphocytes therapies

Tumor-infiltrating lymphocytes (TIL) are extracted from tumors and administered to the patients after *ex vivo* expansion (59, 60). TIL had reproducible effects in melanoma. In a phase 3 trial by Rohaan et al. in 2022, TIL therapy demonstrated an ORR of 49% (41/84) in advanced melanoma (61). There has not yet demonstrated satisfactory efficacy in other solid tumors.

In 2021, Mullinax et al. investigated a rapid expansion protocol that TIL cultures from soft tissue sarcoma resection can expand enough for clinical adoptive cell therapy, which led to an ongoing Phase I trial (NCT04052334) (62).

Current challenges for TIL therapies include high cost due to the personalized nature of TIL therapies, and toxicities from high-dose IL-2, which is given post-TIL administration (63, 64).

4 Cancer vaccines

Talimogene laherparepvec (T-VEC) is an oncolytic viral immunotherapy *via* intratumor injection. It enhances immunogenicity *via* antigen presentation and tumor-specific T cells. T-VEC is the first viral immunotherapy approved for metastatic melanoma (65).

In a Phase II trial, 20 patients with advanced/metastatic sarcoma were treated with an oncolytic virus, T-VEC, with pembrolizumab, which demonstrated an ORR of 35% and a median duration of response of 56.1 weeks (66).

Vaccine therapies have been explored for decades without satisfactory results, likely due to suppressive tumor microenvironment. Current efforts are utilizing novel vectors to promote specificity and strength of immune response.

A novel study by Somaiah et al. demonstrated the efficacy of LV305, a lentivirus vector designed to induce NY-ESO-1 in dendritic cells *in vivo*, improving immune response against tumor cells (67). ORR was 4.2% in sarcoma (1/24 in SS).

CMB305 (a heterologous vaccine for NY-ESO-1 and TLR 4 agonist) is a good vehicle for synovial sarcoma and myxoid/round cell liposarcoma patients, and it was subsequently assessed in a Phase Ib study (68, 69). The study demonstrated a disease control rate of 61.9% and OS of 26.2 months in 64 sarcoma patients. Phase II study with CMB305 and atezolizumab (PD-L1 antibody) compared to atezolizumab alone in STS did not reveal significant improvement in PFS or OS compared to atezolizumab alone (70).

5 Future directions

Although adoptive cellular therapies offer potential individual treatments, they are still in their infancy for soft tissue sarcoma. Targeting fusion-derived cancer testis antigens such as NYESO-1 and MAGEA-4 has shown benefits in limited sarcomas such as synovial sarcoma and Myxoid/Round Cell Liposarcoma (71–73).

Colony-stimulating factor-1 (CSF1) promotes "macrophage polarization", increasing M2/M1 macrophage ratio. CSF1R inhibitor can be a potent immunomodulator by prohibiting the recruitment of TAMs into TME (74). CSF1R-targeting agents have shown a relatively tolerable safety profile but only modest clinical activity.

TTI-621 is a recombinant fusion antibody for SIRPα, a binding domain for CD47, which interrupts inhibition of macrophage phagocytosis mediated by CD47 and stimulates phagocytosis. Combination of doxorubicin with TTI-621 (anti-CD47 antibody) has shown anti-tumor effect in animal models, especially in tumors which express high number of CD47 and macrophages, such as leiomyosarcoma (75). Phase I/II study with TTI-621 alone and in combination with doxorubicin for patients with advanced leiomyosarcoma is underway (NCT04996004).

DR5 Agonist Antibody targeting the TRAIL-TNF axis, which promotes tumor-specific apoptosis, is evaluated in a Phase II study of chondrosarcomas (NCT04950075). NK cell therapies have limited data in solid tumors, and trials for sarcoma (NCT01875601, NCT02890758, NCT03420963) are currently in Phase I.

Envafolimab is a single-domain PD-L1 antibody and administered subcutaneously. There is an ongoing phase II trial evaluating envafolimab alone and with ipilimumab in undifferentiated pleomorphic sarcoma or myxofibrosarcoma (ENVASARC, NCT04480502). A multicenter phase II trial of paclitaxel alone and with nivolumab in taxane-naïve angiosarcoma patients is ongoing. (Alliance A091902, NCT04339738).

In recent years, nanotechnology has shown potential in sarcoma treatment thanks to the development of smart materials and more effective drug delivery systems. Examples include effective docetaxel-loaded mPEG-PLA nanoparticles in sarcoma-bearing mice and albumin-paclitaxel (nab-paclitaxel/Abraxane™) in osteosarcoma mice (76–78). (79) Only four nano-drug delivery systems have been FDA-approved for sarcoma - Doxil (Caelyx)® for AIDS-related Kaposi's sarcoma, DaunoXome® and Lipo-Dox® for Kaposi's sarcoma and Liposomal mifamurtide (MEPACT) for Osteosarcoma. For locally advanced STS, there was a randomized, controlled Phase II-III trial by Bonvalot et al. in 2019 which investigated the role of NBTXR3, a radiation-enhancing nano-particle with radiotherapy compared to radiotherapy alone, demonstrated the efficacy of NBTXR3 with radiation (CR 16% vs. 8%, p = 0.044). There already exists pre-clinical evidence in 2014 which demonstrated that the chitosan nanoparticle-Methylglyoxal complex has effective antitumor properties and elicits macrophage-mediated immunity in Sarcoma-180 tumor-bearing mice (80). A Phase I trial with BO-112 (a synthetic RNA conjugated with nano-sized polyethyleneimine, which activates the immune system) with nivolumab before surgery for resectable STS, is active since 2020. (NCT04420975)

The immunosuppressive microenvironment in STS should be easier to overcome with safer and more effective next-generation immunotherapy. It is currently understood that MMR deficiency is rare and tumor mutation burden is low (3.3/Mb) in STS (7, 81–84). In addition to a traditional concept of "immunologically hot" sarcoma with complex karyotypes which expresses high immune-infiltrate TME and responds well to immunotherapy, there is emerging evidence of epigenetic modulation of transcription in sarcoma, which boosts immunogenicity (85, 86). In a retrospective study of 35 patients, DNA methylation degree correlated with response to anti-PD-1 therapy in sarcoma (87).

There remains a question of whether the mutational burden or neoantigen in STS is clinically correlated to treatment response in immunotherapy. Tumor-infiltrating lymphocytes and PD-L1 expression in STS have shown conflicting prognostic significance thus far. Advancements in bioinformatics and molecular technology will guide the finding of potential biomarkers, which will help fine-tune more effective combinations for each patient in future trials.

6 Summary

Advanced soft tissue sarcoma is still a devastating diagnosis, and there are limited treatments that have long-term success rates.

This article reviewed current immunotherapy in STS, mainly immune checkpoint inhibitors alone or with additional local/systemic therapy and adoptive cell therapy, which modifies the immunogenicity of tumors and TME.

There is a dire need to identify genetic and clinical indicators of response, resistance, and toxicity in immunotherapy in STS. To better characterize histologic/molecular subtypes of STS, tissue and liquid biopsies should be more frequently utilized.

Advancement in the laboratory and clinical immunotherapy of STS for the last five years has been encouraging. By learning from each patient in clinical trials, we hope that patients with soft tissue sarcoma can benefit in the new era of immunotherapy.

Author contributions

GS writing - original draft and editing. SD conceptualization, review, and supervision. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Case report: Epithelioid inflammatory myofibroblastic sarcoma treated with an ALK TKI ensartinib

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Epithelioid inflammatory myofibroblastic sarcoma (EIMS) is an aggressive variant of inflammatory myofibroblastic tumor (IMT) and has a poor prognosis. EIMS is characterized by epithelioid morphology, neutrophilic infiltrate and specific fusion partners of anaplastic lymphoma kinase (*ALK*). Despite no standard therapy for EIMS, ALK tyrosine kinase inhibitors (TKIs) are recommended for these tumors. The present case describes an abdominal mass that presented in a 31-year-old male. The patient suffered from recurrence and multiple metastases 2 months after surgery. Ensartinib was administered and *RANBP2-ALK* fusion was detected. A partial response has been observed for 4 months and there has been no recurrence. This study provided a successful case with sustained response of targeted therapy.

KEYWORDS

epithelioid inflammatory myofibroblastic sarcoma, inflammatory myofibroblastic tumor, *RANBP2-ALK*, ensartinib, fluorescence *in situ* hybridization

Introduction

Inflammatory myofibroblastic tumor (IMT) is as an intermediate soft tissue tumor composed of myofibroblast-differentiated spindle cells along with numerous inflammatory cells, plasma cells, and/or lymphocytes (1). Epithelioid inflammatory myofibroblastic sarcoma (EIMS) is a rare subtype of IMT that is characterized by aggressiveness, rapid local recurrence, earliest metastasis, and fatality (2, 3). EIMS differs from the conventional spindle-cell IMT in that it consists mostly of round-to-epithelioid cells, with a loose or myxoid stroma that is infiltrated with abundant neutrophils (4–7). Among 11 cases, all tumors were located in the abdomen and most originated in the mesentery or omentum (2). In addition to abdomen, several cases with extra-abdominal sites of EIMS have been reported, including liver (8), lung (7, 9), pericardium (10), ovary (11), cutaneous (12), stomach (13), groin (14) and central nervous system (15). A variety of gene partners have been observed in IMT including NPM, TMP3/4, CARS, CLTC, EML4, DCTN1, SEC31L1,

ATIC and FN1 (5), with more prevalent fusions of RANBP2 (2), RRBP1 (16) and EML4 (5) in EIMS. Furthermore, G1269A has been reported as a secondary mutation after resistance to crizotinib (17). As described herein, a patient with *RANBP2-ALK* EIMS in the greater omentum benefited from an ALK TKI.

Case description

An intermittent abdominal pain and abdominal distention were reported by a 31-year-old Chinese male. Enhanced computed tomography (CT) scans of the abdomen revealed an abdominal mass that was suspected to be gastric stromal tumor (Figure 1 and Table 1). Biopsy showed a loose tissue composed of spindle cells, hollow cells and small blood vessels. Fluorescence *in situ* hybridization (FISH) was negative for CHOP and MDM2, excluding the presence of liposarcoma. Abdominal tumor resection plus partial colectomy was then performed in March 2022, removing the greater omentum tumor as well as partial transverse colon and ascending colon with a volume of 12 × 10 × 10 cm. The histopathological results revealed the lesion contained both epithelioid and spindle cells with enlarged nuclei and infiltrating inflammatory cells, primarily plasma cells, eosinophils, neutrophils, lymphocytes (Figures 2A, B). EIMS usually expressed vimentin and desmin positively, the Expression situation of EMA, CD30 and SMA was inconsistent. EIMS can be distinguished by IHC from soft tissue tumors with epithelioid cell morphology and tumors with significant mucoid background. Such as anaplastic large cell lymphoma (ALCL) and EIMS were positive for SMA, CD30 and ALK and negative for EMA (29). But no desmin was found in ALCL. Follicular dendritic cell sarcoma (FDCS): Immunohistochemical expression of CD21, CD23, or CD35 was positive, but no ALK,Desmin,WT-l,orD2-40 (30). Extragastrointestinal stromal tumor (EGIST) CD117, CD34 and Dog-1 were positive, and ALK;CKDesmin were negative (31). Therefore, the following markers were selected for immunohistochemistry, and the results were as follows. ALK, Vimentin, Desmin, SAM, Ki-67, CD30, CD31, Catenin and H3K27Me3 were positive, while negative for Cytokeratin (CK), CD34, CD117, S-100, SOX-10, Dog-1, Muc-4, EMA and ERG (Table 2). Antibody clone of ALK-IHC is ALK p80(5A4), and Positive immunohistochemical results of ALK, CD30, desmin, SAM and Vimentin are shown in turn in this Figures 2C–G. Approximately 18% of the tumor cells showed a rearrangement of ALK by FISH (Figure 2H). These findings are in line with those of EIMS. Adjuvant therapy was not administered to the patient following surgery.

The patient presented to our hospital with abdominal distension 74 days after surgery. CT showed new soft tissue mass with peritoneal metastasis in lower abdomen and pelvis, indicating tumor recurrence. The tumor was significantly enlarged 14 days later by CT scan. The therapeutic course and radiological examinations of the patient were summarized in Figure 1. The patient was subsequently treated with ensartinib (225 mg, QD). The CT revealed notable tumor shrinkage 13 days after ensartinib treatment. In September 29, 2022, 4 months after initiation of ensartinib, this patient still achieved partial response (PR) according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (Figure 1). Next-generation sequencing (NGS) was employed using a 1021-gene panel (Burning Rock, Guangzhou, China). NGS identified *RANBP2-ALK* fusion (EX18:EX20) with mutational abundance of 2.3% and a TP53 p.R282W mutation with a mutational abundance of 3.2% using the biopsy specimen of the abdominal mass (data not shown). As we prepared the manuscript, the patient remained PR and continued to receive ensartinib without any significant adverse events.

Discussion

EIMS is first named in 2011 (2) and is a more aggressive subtype of IMT and characterized by epithelioid-to-round cell morphology and prominent inflammatory infiltrate. EIMSs can occur across a wide age range (4 months to 76 years), with a male and intra-

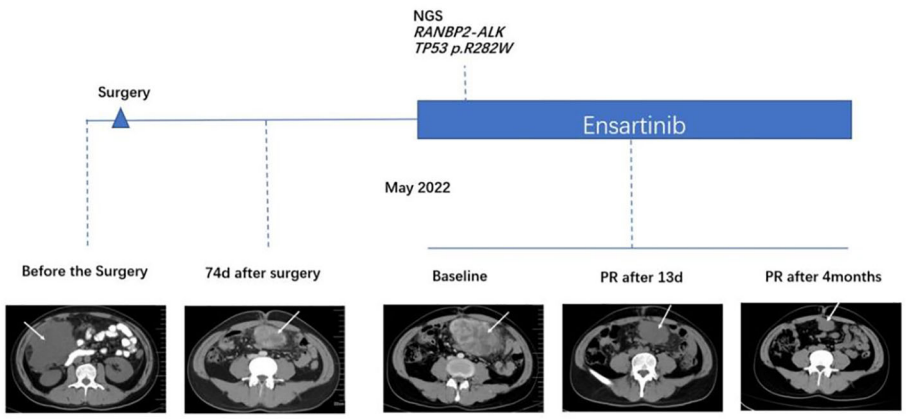

FIGURE 1
Comparison of computed tomography images before and after treatment with ensartinib.

TABLE 1 Clinicopathological characteristics of EIMS in reported cases and our case.

Case/Reference	Age/Sex	Site	Symptom	Size (cm)	Multifocal	Treatment	Specific drug names of ALK-TKIs	Response at TKIs	PFS at TKIs (m)	Follow-up (m)
1 (18)	44/M	Omentum	early satiety and abdominal pain	NA	Y	SE+CT+ALKi→ALKi→ALKi	Imatinib→Crizotinib→Crizotinib	NA→PR→PR	3→8→19 (AWD)	40 (AWD)
2 (2)	41/M	Omentum	NA	26	Y	SE+CT+ALKi	NA	NA	NA	40 (ANED)
3 (19)	57/M	Pleura or chest wall	dyspnea on exertion	NA	NA	ALKi	NA	NA	NA	NA
4 (20)	22/M	the ileum	fever, epigastralgia	5.5×6	Y	SE+CT+ALKi	crizotinib	PR	10(AWD)	17 (AWD)
5 (7)	21/M	the left lower lobe of lung	general fatigue and rapid weight loss	10	Y	SE+ALKi	crizotinib	NA	4	4(STD)
6 (21)	71/M	the lung and pleural-based mass	dyspnea on exertion and weight loss	12.5×12×8	Y	SE+CT+ALKi →ALKi	crizotinib→NA	NA→PR	2→9 (AWD)	12 (AWD)
7 (22)	16/F	the lung	NA	8	NA	SE+CT+RT+ALKi	crizotinib	PR	19 (AWD)	33 (AWD)
8 (23)	22/M	the transverse colon mesentery	abdominal pain and fever	13	NA	SE+ALKi	crizotinib	NA	16 (AWD)	17 (AWD)
9 (24)	22/M	Mesentery of colon	abdominal pain and fever	20×15	Y	SE+ALKi	crizotinib	PR	12 (AWD)	14 (AWD)
10 (5)	45/M	omentum	abdominal distention and abdominal pain	20	Y	SE+ALKi	crizotinib	NA	0.5	2(STD)
11 (11)	15/F	Ovary	NA	NA	Y	CT+SE+ALKi	crizotinib/ceritinib	PR	NA	24 (AWD)
12 (17)	26/M	Abdomen	fever, abdominal distention	NA	Y	ALKi→ALKi	crizotinib→brigatinib	PR→PR	9→8(AWD)	24 (AWD)
13 (25)	46/F	Abdomen	abdominal pain, abdominal distention	11×6.5×7	NA	SE+ALKi+CT	crizotinib	PR	2	14(STD)
14 (26)	NA	colon sigmoideum	NA	11.9×6.9	NA	SE+ALKi	crizotinib	NA	NA	NA
15 (15)	72/F	brain	NA	4.7×1.6×1.2	N	SE+ALKi	Alectinib	NA	NA	NA
16 (27)	42/F	omentum	abdominal distention and abdominal pain	19×19×10	Y	SE +ALKi→ALKi→ALKi→ALKi	Crizotinib→Alectinib→Ceritinib→Lorlatinib	PR→PR→PR→SD	5→5.5→6→>5 (AWD)	>24 (AWD)

(Continued)

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TABLE 1 Continued

Case/Reference	Age/Sex	Site	Symptom	Size (cm)	Multifocal	Treatment	Specific drug names of ALK-TKIs	Response at TKIs	PFS at TKIs (m)	Follow-up (m)
17 (28)	14.7/M	pelvis	Ascites, pleural effusions	NA	N	ALKi	crizotinib→crizotinib	CR→PR	5→17	NA
18 (28)	11.3/M	Abdomen mesenteric	NA	NA	Y	SE+ALKi	crizotinib	PR	48 (ANED)	48 (ANED)
19 (28)	9.1/M	Abdomen mesenteric	Massive ascites, abdominal compartment syndrome	NA	Y	ALKi→ALKi	crizotinib→ceritinib+CT	CR→PR	11→6	23(STD)
20 (28)	1.4/F	Abdomen mesenteric	NA	NA	Y	ALKi	crizotinib	CR	11(AWD)	11 (AWD)
Current case	**31/M**	**Abdomen**	**abdominal distention and abdominal pain**	**12×10×10**	**Y**	**SE+ALKi**	**Ensartinib**	**PR**	**4(AWD)**	**4(AWD)**

F, female; M, male; Y, yes; N, no; m, month; cm, centimeter; PFS, progression-Free-Survival; ALKi, ALK inhibitor; ANED, alive, no evidence of disease; AWD, alive with disease; STD, succumbed to disease; CT, chemotherapy; NA, data not available; SE, surgical excision; CR, complete response; PR, partial respond; SD, stable disease.
The bold values indicate the current case.

abdominal predominance (15). Recently, IMTs associated with *EML4-ALK* have been classified as EIMS (5).

EIMS is essentially a histopathological diagnosis. Even with auxiliary detection, it is difficult to make a definitive diagnosis of EIMS on a small biopsy due to its genetic overlap with other ALK-positive tumors. To date, more than 40 cases of EIMS have been reported, among which 20 cases (2, 5, 7, 11, 15, 17–28) as well as present case with the complete clinicopathological, immunohistochemical and genetic characteristics were summarized in Table 1, 2. These 21 cases were composed of 14 (67%) adults (21-72 years old) and 6 (29%) children or adolescents. The median age was 24 years old. Fourteen (67%) patients were male while six (29%) were female. In 16 patients, the tumors were found in abdominal cavity (omentum, mesenterium, ileum, colon, ovary, etc.), 4 in pleural cavity and 1 in brain, which consisted with previous reports that EIMS had a male and intra-abdominal predilection. In contrast, conventional spindle-cell IMT was slightly more prevalent among females. The EIMS exhibits distinctive morphological characteristics including loosely arranged round or epithelioid neoplastic cells with vesicular nuclei, prominent nucleoli and myxoid stroma surround by amphophilic to eosinophilic cytoplasm. Neutrophil-rich inflammatory infiltrates are a striking characteristic of EIMS. Almost all tumors contained a spot of spindle cell component.

According to immunohistochemical results in 15 cases with EIMS, 8 (53%) revealed a unique nuclear membrane staining pattern for ALK (Table 2). Nevertheless, cytoplasmic or perinuclear staining of ALK was observed in 7 of 15 cases (47%). All cases (13/13) exhibited strong expression of desmin, another diagnostic immunophenotype. Besides, the tumor displayed variable expression of CD30 (69%, 9/13), alpha smooth muscle actin (33%, 5/15) and epithelial membrane antigen (13%, 1/8). Moreover, all cases were negative for cytokeratin (0/9), myogenin (0/7), anoctamin−1 (0/4) and S-100 (0/11). Of note, FISH assay, PCR assay or NGS can contribute to the diagnosis of EIMS. It has been confirmed that ALK rearrangement is present in 16 cases by FISH, 8 cases by PCR and 4 cases by NGS.

RANBP2-ALK and *RRBP1-ALK* fusions were the most reported driver mutation of EIMS (16, 32). In previous studies as well as our case with EIMS, nuclear membrane or perinuclear staining pattern for *RANBP2-ALK* fusion was detected, and almost all cases containing *RANBP2-ALK* fusion exhibited aggressive behavior (2, 12, 18, 20, 33–35). Despite of the unclear biological function, the chimeric *RANPB2-ALK* gene was reported to promote cell growth and proliferation regardless of cytokine *in vitro* (36, 37). There was a specificity for *RRBP1-ALK* in EMIS with cytoplasmic ALK expression and clinically aggressive progression, suggesting that *RRBP1-ALK* may exert relapsed oncogenic role in clinically aggressive EIMS (16). Recently, *PRRC2B-ALK* fusion was also considered as the main oncogenic driver of the EIMS (27).

There is no clear consensus on the best treatment for EIMS. The main treatment option remains surgical resection. Postoperative adjuvant therapy has not yet been identified due to the limited available experiences. In terms of rapid recurrence, postoperative chemotherapy or radiotherapy appeared to exert finite effect (2, 18, 20, 35). Crizotinib has been applied to treat EIMS in several cases

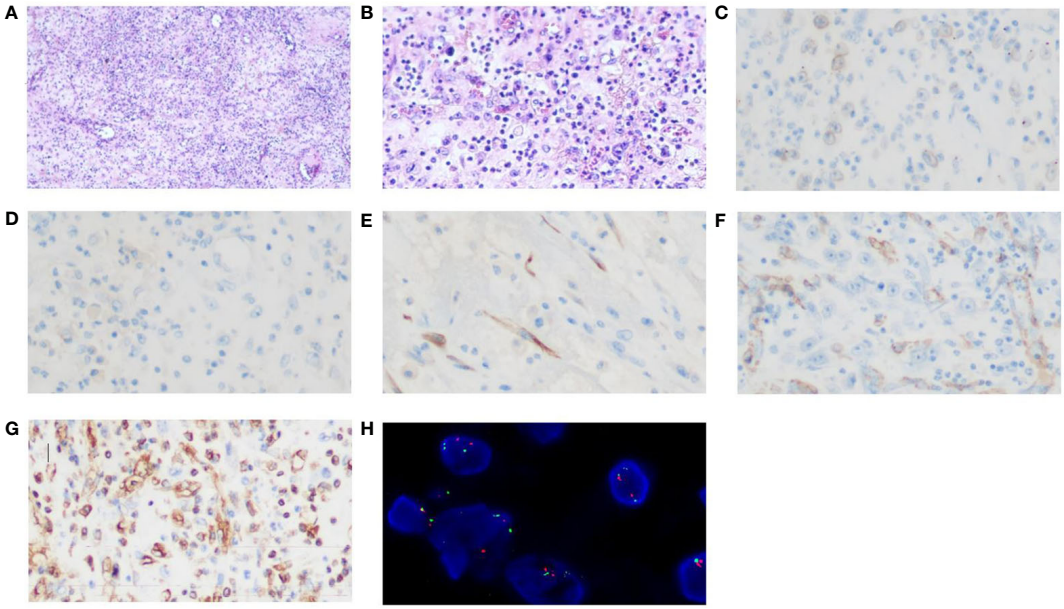

FIGURE 2
Histopathological examination with haematoxylin and eosin staining and Immunohistochemistry and FISH images. The lesion consisted of both epithelioid and spindle cells with enlarged nucleolus and inflammatory cells infiltration **(A)** (magnification, x40). **(B)** (magnification, x200). **(C)** ALK positivity (magnification, x400); **(D)**Focal CD30 weakly positive (magnification, x400); **(E)**Desmin positivity (magnification, x400) **(F)**SMA positivity (magnification, x400) **(G)** Vimentin positivity (magnification, x400); **(H)** A FISH image of ALK rearrangement.

and showed favorable efficacy (2, 18–20, 38). In our summarized cases, 10 patients continued to live with disease with follow-up of 11-40 months. Among 21 patients, 17 received crizotinib, apart from 2 unknown TKIs, 1 ensartinib and 1 alectinib. Fifteen of 17 patients who received crizotinib had survival information, with a median PFS of 9 months and mean PFS of 10.8 months. Furthermore, a case with *PRRC2B-ALK* fusion showed durable clinical response to sequential use of ALK TKIs (crizotinib, alectinib, ceritinib and lorlatinib) (27). The development of drug resistance to ALK TKIs is a major issue. A previous case revealed that R1192P was found as a resistance mutation to crizotinib (27), suggesting that the application of NGS was important to identify actionable mutations and resistance mechanisms. This may contribute to molecular targeted therapies for EIMS with *ALK* gene arrangement. Considering the broader coverage of targets and stronger tissue penetration, we speculated that patients may have more benefits from the direct second- or third-generation ALK TKIs compared with the sequential treatment of ALK TKIs. Therefore, in our own case, the patient received ensartinib (a second-generation ALK TKI) post relapse. After four months of treatment, the patient felt well with PR, and follow-up CT scan showed that the residual tumor was partially shrank.

Recently, a diffuse positive signal was observed in EIMS for programmed death-ligand 1 (PD-L1) (4), providing a possible inmmunomodulatory therapies targeting the PD-1/PD-L1 pathway. Moreover, CD30 appeared to commonly express in EIMS in our summarized cases (Table 1). The survival time was prolonged by ALK and CD30 combination therapies (39). With increasing evidence of EIMS, molecular mechanisms will be clear and potential treatments will be developed in the future.

In addition to *RANBP1-ALK* fusion, TP53 p.R282W mutation was also identified in our case. Mutant TP53 is closely related to the occurrence, development and prognosis of tumor (40, 41). Besides, mutations in TP53 independently promoted metastasis, decreased TKI responses and shorten overall survival in ALK-positive lung adenocarcinoma (42). However, the molecular pathological importance of the TP53 mutation in IMT has not been elucidated thus far. A previous study demonstrated that abnormal TP53 staining patterns were detected in only approximately 7% of IMT, with TP53 missense mutations occurring in 13% of cases, suggesting that TP53 mutation in IMT was an infrequent event and may not attribute to its pathogenesis (43). Recently, a study demonstrated that ORR of ensartinib was high regardless of TP53 mutation status (44). In our case, ensartinib also showed favorable efficacy.

Conclusion

In conclusion, we described a typical EIMS case with a round or epithelioid morphology of cells, accompanied by a high relapse and a poor prognosis. To the best of our knowledge, our report is the first case to investigate the efficacy of ensartinib for EIMS. The clinical management and results of the patients were introduced in detail in our case; besides, the pathological and genetic characteristics of the tumors were reviewed. By analyzing the

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TABLE 2 Immunohistochemical and genetic characteristics in reported cases and our case.

Case	Vimentin	DES	SMA	CD30	CK	EMA	MYF4	DOG 1	S-100	ALK			
										IHC	FISH	RT-PCR	NGS
1	NA	+	–	NA	–	NA	–	NA	NA	NM	ALK-rearrangement	RANBP2	NA
2	NA	+	–	+	–	–	–	NA	–	NM	ALK-rearrangement	RANBP2	NA
3	+	+	–	–	–	NA	–	NA	–	PN	RANBP2	NA	NA
4	NA	+	+	NA	NA	NA	–	NA	–	NM	NA	RANBP2	NA
5	+	+	–	–	–	NA	–	NA	–	CN	ALK-rearrangement	NA	NA
6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	ALK-rearrangement	NA	NA
7	NA	NA	NA	+	NA	NA	NA	NA	NA	CN	ALK-rearrangement	NA	NA
8	NA	+	–	+	–	NA	NA	–	–	PN	RANBP2	NA	NA
9	NA	+	–	–	–	–	–	NA	–	CN	ALK-rearrangement	NA	NA
10	NA	+	+	NA	NA	NA	NA	NA	NA	CN	NA	EML4	NA
11	NA	+	+	NA	NA	NA	NA	NA	NA	NM	NA	NA	RANBP2
12	NA	NA	–	NA	–	–	–	NA	NA	NA	ALK-rearrangement	NA	RANBP2
13	+	NA	+	NA	NA	NA	NA	–	–	NA	NA	NA	NA
14	NA	+	–	+	–	–	NA	–	–	NA	RRBP2	NA	NA
15	NA	+	–	–	–	+	NA	NA	–	CN	NA	VCL	NA
16	NA	+	–	NA	–	–	NA	NA	–	NA	ALK-rearrangement	NA	PRRC2B
17	NA	NA	NA	+	NA	NA	NA	NA	NA	NM	ALK-rearrangement	RANBP2	NA
18	NA	NA	NA	+	NA	NA	NA	NA	NA	NM	ALK-rearrangement	RANBP2	NA
19	NA	NA	NA	+	NA	NA	NA	NA	NA	NM	ALK-rearrangement	RANBP2	NA
20	NA	NA	NA	+	NA	NA	NA	NA	NA	NM	ALK-rearrangement	NA	NA
Current case	+	+	+	+	-	-	**NA**	-	-	**NA**	**ALK-rearrangement**	**NA**	**RANBP2**
Total	100%	100%	33%	69%	0%	13%	0%	0%	0%				
	(4/4)	(13/13)	(5/15)	(9/13)	(0/9)	(1/8)	(0/7)	(0/4)	(0/11)				

+, positive cells; –, negative staining; NM, nuclear membrane staining; PN, cytoplasmic staining with perinuclear accentuation; CN, cytoplasmic pattern; CK, cytokeratins; DES, desmin; EMA, epithelial membrane antigen; FISH, fluorescence in situ hybridization; MYF4, myogenin; CD, cluster of differentiation; DOG-1, anoctamin–1; NGS, next-generation Sequencing; NA, data not available; RT–PCR, reverse transcription–polymerase chain reaction; SMA, smooth muscle actin; IHC, immunohistochemistry; ALK, anaplastic lymphoma.
The bold values indicate the current case.

efficacy of ALK TKIs in EIMS in previous literature, we found that ALK TKIs are effective for EIMS treatment. However, given the lack of the clear resistance mechanisms, further research is needed. Detection of *ALK* rearrangement is essential for correct diagnosis of EIMS and provides a fundamental basis for ALK TKI therapy.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Henan Cancer Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

>HW contributed to the conception of the study. ML and RX integrated all information and wrote the main manuscript. CW supervised the writing process. CS and JH provided critical guidance. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Formulation and validation of a baseline prognostic score for osteosarcoma treated uniformly with a non-high dose methotrexate-based protocol from a low middle income healthcare setting: a single centre analysis of 594 patients

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Introduction: The outcomes of osteosarcoma in low middle income countries (LMICs) are different due to patients presenting in advanced stages, resource constraints and the use of non-high-dose-methotrexate (HDMTX)-based regimens. This study derived and validated a prognostic score for osteosarcoma that integrates biologic and social factors and is tailored for patients from an LMIC setting using a non-HDMTX-based protocol.

Materials and methods: A retrospective study including osteosarcoma patients enrolled for treatment at a single tertiary care centre in India between 2003-19 was conducted. Baseline biologic and social characteristics were extracted from medical records and survival outcomes were noted. The cohort was randomised into a derivation and validation cohort. Multivariable Cox regression was used to identify baseline characteristics that were independently prognostic for survival outcomes in the derivation cohort. A score was derived from the prognostic factors identified in the derivation cohort and further validated in the validation cohort with estimation of its predictive ability.

Results: 594 patients with osteosarcoma were eligible for inclusion in the study. Around one-third of the cohort had metastatic disease with 59% of the patients residing in rural areas. The presence of metastases at baseline (HR 3.39; p<0.001; score=3), elevated serum alkaline phosphatase (SAP) >450 IU/L (HR 1.57; p=0.001; score=1) and baseline tumour size > 10 cm (HR 1.68; p<0.001; score=1) were

identified to be independent factors predicting inferior event free survival (EFS) and were included in development of the prognostic score. Patients were categorized as low risk (score 0), intermediate risk (score 1-3) and high risk (4-5). Harrell's c-indices for the score were 0.682, 0.608 and 0.657 respectively for EFS in the derivation, validation and whole cohort respectively. The timed AUC of ROC was 0.67 for predicting 18-month EFS in the derivation, validation and whole cohorts while that for 36-month EFS were 0.68, 0.66 and 0.68 respectively.

Conclusions: The study describes the outcomes among osteosarcoma patients from an LMIC treated uniformly with a non-HDMTX-based protocol. Tumor size, baseline metastases and SAP were prognostic factors used to derive a score with good predictive value for survival outcomes. Social factors did not emerge as determinants of survival.

KEYWORDS

prognostic, score, osteosarcoma, low middle income countries, bone sarcoma

Introduction

Osteosarcoma is the most common bone sarcoma worldwide (1, 2). The survival rates for bone sarcomas have improved over the last two decades on account of the incorporation of multi-modality treatment regimens. However, treatment outcomes continue to lag behind in low and low middle income countries (LMICs) due to a multitude of factors (2, 3). In LMICs, patients tend to present at advanced stages with high disease burden at presentation. Furthermore, healthcare accessibility, surgical expertise, access to good supportive care, treatment abandonment rates and compliance to treatment remain poorer in LMICs (4, 5). While high-dose-methotrexate(HDMTX)-based protocols have become the standard chemotherapy regimens in resource-rich settings, the delivery of HDMTX-based regimens entails logistic difficulties in the form of need for inpatient admission and strong supportive care, thus necessitating the use of alternate strategies in settings with resource limitations (6). Thus, treatment outcomes and their determinants are likely to be different in LMICs.

The identification of prognostic factors at baseline may facilitate tailoring of therapy based on disease risk. Prior studies have explored prognostic factors for survival in osteosarcoma. Baseline clinical factors such as extremes of age, large tumour sizes, axial tumour site as opposed to appendicular, and the presence of metastases have been found to be associated with worse survival outcomes (7–12). In addition, baseline lab parameters such as the neutrophil-lymphocyte ratio and alkaline phosphate have also been described to be of prognostic significance (13, 14). Among tissue immunohistochemistry markers assessed for prognostic value, tumour vascular endothelial growth factor (VEGF) response to neoadjuvant therapy has been noted to predict for more aggressive disease biology, while tumour HER2/neu expression was not found to be prognostic (15, 16).Imaging response surrogates using ^{18}F-fluorodeoxyglucose positron emission tomography, computed tomography (^{18}F-FDG PET-CT) and dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) have been evaluated as markers for response to neoadjuvant chemotherapy (17, 18). Patients with poor histopathologic response to neoadjuvant therapy have been described to have inferior treatment outcomes (8, 19, 20). However, the intensification of therapy based on necrosis has not been conclusively shown to improve survival, especially among patients receiving HDMTX-based protocols (21). Therapy intensification based on baseline perceived disease risk has not been attempted previously on a background of chemotherapy protocols used in the current era (22, 23). The studies from which prognostic markers have been identified are largely registry based or have evaluated patients enrolled in large randomised controlled trials, which may not be reflective of the real world scenario. Furthermore, there is a striking lack of data from LMICs on therapeutic outcomes in osteosarcoma, wherein treatment protocols and the challenges involved in implementing them are unique.

In resource-challenged settings, social factors are also significant contributors to treatment outcomes. We have previously seen that the magnitude of gender disparity in seeking treatment for childhood cancer was dependent on the cost involved (24). Studies from the West have noted that social factors such as socioeconomic status and the possession of health insurance may be major determinants of survival in osteosarcoma (25, 26). Since the influence of social factors is likely to be more apparent in an LMIC

Abbreviations: LMIC, low middle income country; HDMTX, high dose methotrexate; VEGF, vascular endothelial growth factor; ^{18}F, FDG PET, CT, ^{18}F, Fluorodeoxyglucose positron emission tomography, computed tomography; DCE, MRI, dynamic contrast enhanced magnetic resonance imaging; MRI, magnetic resonance imaging; NCCT, non, contrast computed tomography; RECIST, Response Evaluation Criteria for Solid Tumors; EFS, event free survival; OS, overall survival; ROC, receiver, operating characteristic curve; AUC, area under curve; HR, hazard ratio; CI, confidence interval.

setting, it is of great importance to identify their contribution to treatment outcomes along with tumor-related biological factors.

This study was conducted to derive and validate a prognostic score based on baseline disease characteristics along with analysis of impact of social characteristics on outcome in patients with osteosarcoma in an LMIC setting treated uniformly using a non-HDMTX based regimen. This may allow clinicians in LMICs to better risk stratify and tailor treatment based on the distinctive characteristics of patients with osteosarcoma hailing from more resource-challenged parts of the world.

Methods

Study design

This is a retrospective study from a single tertiary care cancer centre in India. Consecutive patients registered in the period between 2003 to 2019 in the medical oncology outpatient department were included. All patients included had a histopathologic diagnosis of osteosarcoma confirmed based on characteristic morphologic features seen on the biopsy specimen and discussion in the interdisciplinary conference. Patients who had received chemotherapy outside prior to or after presentation to our centre and those lost to follow up after receiving less than two cycles of (neo)adjuvant chemotherapy at our centre were excluded. Ethics approval was taken from the institute ethics committee (IEC-454/06.05.2022, RP-34/2022). In view of the retrospective nature of the study, the need for informed consent was waived off.

Data collection

For all included patients, treatment files were reviewed to collect baseline data. Telephonic follow up was done to enhance data retrieval for patients with missing data and for those who were lost to follow up. Baseline clinical characteristics such as age, gender, symptom duration prior to presentation, presence of fever, clinical evidence of neurovascular bundle involvement, tumour size and disease stage were recorded. The baseline lab parameters compiled included hemogram and liver and renal function tests including serum alkaline phosphatase. The social characteristics comprised distance of the patient's residence from the treating centre and the type of residence (rural versus urban). GoogleMaps was used to derive the distance of the treating centre from the address (27). The place of residence was categorised as rural or urban based on the address as per the National Census 2011 (28). Patients with metastatic disease were classified as "limited burden metastases" if they had two or fewer lung metastases and those with 3 or more lung metastases or any extrapulmonary metastases were classified as "extensive metastases".

Evaluation of the patient at baseline

All patients with confirmed diagnosis of osteosarcoma availing treatment at our institute were subjected to a standard set of baseline investigations prior to initiation of treatment. Imaging of the local site was done with MRI (magnetic resonance imaging). Baseline staging was done using either ^{18}F-FDG PET-CT of the whole body or with a combination of non-contrast computed tomography (NCCT) of the thorax and a 99m-technetium methylene diphosphonate (Tc-99m MDP) bone scintigraphy.

Treatment protocol

All patients were treated with a uniform non-HDMTX-based chemotherapy protocol. Three cycles of neoadjuvant therapy with cisplatin and doxorubicin were administered following which therapy response was evaluated with the help of local and distant site imaging. The RECIST 1.0 criteria were used for response assessment. Local therapy was planned after multidisciplinary discussion with the surgical team. The histopathologic response to neoadjuvant therapy was assessed based on necrosis in the postoperative specimen. Patients showing good responses (necrosis > 90%) were given three cycles of adjuvant chemotherapy with cisplatin and doxorubicin; on the other hand, patients with poor responses (necrosis < 90%) were given three alternating cycles each of cisplatin/doxorubicin and ifosfamide/etoposide as adjuvant chemotherapy (19, 29, 30). In patients with lung metastases at baseline, patients with partial or complete responses following neoadjuvant chemotherapy were considered for lung metastasectomy. Patients with disease progression at the metastatic site(s) were managed further with palliative intent.

Outcomes of the study

The primary outcome in our study was event free survival (EFS) and the secondary outcome was overall survival (OS). The EFS was defined as the time between initiation of treatment and either disease progression or death from any cause. OS was defined as the time between treatment initiation and death from any cause. The data was censored on 30 November 2022.

Statistical analysis

Statistical analysis was done with the help of STATA v.17 (StataCorp, College Station, TX, USA). Descriptive statistics was used to summarize baseline characteristics. Continuous variables were represented by median with range. The chi-square test and Mann-Whitney test were used to compare categorical and continuous variables respectively, and Kaplan Meier analysis was done along with log rank test to compare time to event outcomes. The follow-up estimation of the cohort was done using reverse Kaplan Meier method. The association of social factors [distance from treating centre (>100 km versus < 100 km) and type of residence (rural versus urban)] with baseline clinical characteristics was analysed by the chi-square test while the impact of social factors on survival outcomes was analysed by the log rank test. The impact of burden of metastases (limited versus extended burden metastases) on survival was also analysed by the Kaplan Meier and Cox regression methods.

Generation of the derivation and validation cohorts and identification of prognostic factors in the derivation cohort

The whole cohort was divided in a 2:1 ratio into a derivation and validation cohort in a randomised fashion. The baseline factors assessed as potential prognostic factors included age (>18 vs ≤ 18 years), gender, symptom duration prior to presentation (>4 months vs ≤ 4 months), presence of fever, disease stage (localised versus metastatic), tumour size (>10 cm vs ≤ 10 cm), tumour site (axial vs appendicular), clinical presence of neurovascular bundle involvement, haemoglobin (<11 g/dL vs ≥ 11 g/dL), total leucocyte count (≤11000/μL vs >11000/μL), serum albumin (≥3.5 g/dL vs < 3.5 g/dL), serum alkaline phosphatase (>450 IU/L vs ≤ 450 IU/L). Univariable cox regression analyses were used to identify baseline factors prognostic for EFS in the derivation cohort. Factors with p-value less than 0.1 on univariable analyses were included for multivariable analysis in a forward stepwise fashion based on likelihood ratio. Factors with $p<0.05$ in the final multivariable model in the derivation cohort were used to formulate the risk score.

Formulation of risk score

A weighted score was provided to each prognostic variable. The score was computed based on the approximate ratios of the beta coefficients of each factor in the multivariable model. The total score was calculated by summation of individual prognostic factor scores and was used to divide patients into three clinically discriminatory risk groups.

Validation of the risk score

The risk score was validated by applying it separately to the derivation, validation, and whole cohorts separately. Kaplan Meier curves were constructed to represent EFS and OS in the three risk groups in each of the three cohorts. Harrell's concordance index (c-index) was calculated for estimating the predictive ability of the risk category model for EFS and OS in the derivation, validation and whole cohorts. A receiver operating characteristic (ROC) curve was also constructed by comparing the predicted and actual 18-month and 36-month EFS and OS in each of the three cohorts and the timed area under the ROC curve (timed AUC) for the derivation, validation and whole cohort was estimated.

Results

Baseline patient characteristics and survival outcomes

During the study period from 2003 to 2019, a total of 640 patients with osteosarcoma registered at our centre with available data records were screened for inclusion in the study, out of which 594 patients were finally included for analysis (Figure S1). The baseline sociodemographic and clinical characteristics of the entire cohort are summarized in Table 1. The median age of presentation was 18 years (range: 2-71 years) with predominantly male patients (411/594; 69.2%) and a male to female ratio of 2.25:1. At presentation, the median tumor diameter (longest dimension) at the primary site was 10cm (range: 1-48 cm) with pathological fracture observed in 126 (21.4%) patients. Baseline metastatic disease was noted in more than one-third (204/594; 34.3%) of patients. At a median follow, up of 51.7 months (35.7-67.7 months), the median EFS of the whole cohort was 17.03 months while the estimated median OS was 80 months. The cohort was randomized 2:1 to yield 396 patients in the derivation cohort and 198 patients in the validation cohort. The baseline clinical and sociodemographic characteristics as well as the survival outcomes were similar between the two groups (Table 1).

Identification of prognostic factors in the derivation cohort

In the derivation cohort, on univariable analysis, the presence of baseline metastatic disease (HR=3.39; $p<0.001$); tumor diameter (longest dimension) >10cm (HR=1.68; p=0.005); neurovascular involvement at the primary site (HR=2.84; $p<0.001$); presence of a pathological fracture at baseline (HR=2.02;$p<0.001$); higher baseline serum alkaline phosphatase (>450 IU/L) (HR=1.57; p=0.001); and baseline anemia (hemoglobin < 11g/dL) (HR=1.38; p=0.021) were predictive of inferior EFS. However, on multivariable analysis, only the presence of baseline metastases (HR=3.55; $p<0.001$); tumor diameter >10cm (HR=1.38; p=0.045) and higher serum alkaline phosphatase (HR=1.50; 95%; p=0.010) were independently predictive of inferior EFS in the derivation cohort (Table 2; Figures 1A–C). The above three factors were also predictive of inferior OS in the derivation cohort. (Figures 1D–F).

Formulation of baseline prognostic risk categories

The three independent prognostic factors predicting inferior EFS in the derivation cohort were used to formulate a baseline prognostic risk score. Based on the ratio of beta-coefficient of the final multivariable Cox regression model, a weighted integer score was assigned to each prognostic factor: presence of metastases (score of 3); tumor diameter >10cm at primary site (score of 1) and baseline serum alkaline phosphatase >450IU/L (score of 1). Based on the scores, the patients were further categorized to clinically discriminatory risk categories (low risk: Score of 0; intermediate risk: score of 1,2 and 3; high risk: score of 4 and 5).

Prognostic ability of the risk score category for event free survival

On application of the risk score to categorise patients in the validation cohort, the median EFS was significantly different among the three risk categories (median EFS of low risk, intermediate risk

TABLE 1 Baseline clinical and socio, demographic characteristics in derivation (n=396), validation (n=198) and whole cohort (n=594).

Clinical/Socio, demographic parameter (median with range)	Categories	Whole cohort (n=594)	Derivation cohort (n=396)	Validation cohort (n=198)	P-value*
*** Clinical/demographic parameters**					
1. Age (years)	Median (range)	18 (2 , 71)	18 (4, 66)	17 (2, 71)	0.499
	≤18 years	344 (57.9%)	225 (56.8%)	119 (60.1%)	
	> 18 years	250 (42.1%)	171 (43.2%)	79 (39.9%)	
2. Sex	Male	411 (69.2%)	270 (68.2%)	141 (71.2%)	0.451
	Female	183 (30.8%)	126 (31.8%)	57 (28.8%)	
3. Metastases	Non-metastatic	390 (65.7%)	265 (66.9%)	125 (63.1%)	0.359
	Metastatic	204 (34.3%)	131 (33.1%)	73 (36.9%)	
4. Tumor diameter of primary tumor (longest dimension) (cm) (n=482)	Median (range)	10 (1-48)	9.4 (1-48)	10.4 (2-29)	0.048
	≤10cm	270 (56.0%)	191 (59.3%)	79 (49.4%)	
	>10cm	212 (44.0%)	131 (40.7%)	81 (50.6%)	
5. Symptom duration (months) (n=502)	Median (range)	4 (1-36)	4 (1-36)	4 (1-36)	0.953
	≤4months	287 (57.2%)	190 (57.2%)	97 (57.1%)	
	>4months	215 (42.8%)	142 (42.8%)	73 (42.9%)	
6. Site of disease (n=525)	Axial	33 (6.3%)	20 (33.1%)	13 (7.5%)	0.431
	Appendicular	492 (93.7%)	331 (94.3%)	161 (92.5%)	
7. Fever at baseline	Yes	60 (10.1%)	34 (8.6%)	26 (13.1%)	0.083
	No	534 (89.9%)	362 (91.4%)	172 (86.9%)	
8. Fracture at presentation (n=590)	Yes	126 (21.4%)	85 (21.6%)	41 (20.8%)	0.820
	No	464 (78.6%)	362 (91.4%)	172 (86.9%)	
9. Neurovascular bundle involvement (n=582)	Yes	111 (19.1%)	67 (17.2%)	44 (22.9%)	0.098
	No	471 (80.9%)	323 (82.8%)	148 (77.1%)	
10. Hemoglobin (g/dL) (n=572)	Median (range)	11.7 (4 – 16.9)	11.8 (4-16.9)	11.5 (4.2-15.7)	0.182
	<11g/dL	208 (36.4%)	138 (36.1%)	70 (36.8%)	
	≥11g/dL	364 (63.6%)	244 (63.9%)	120 (63.2%)	
11. Total leucocyte count (/μL) (n=571)	Median (range)	8300 (990-42800)	8250 21-42800	8300 (990-24800)	0.930
	≤11000	481 (84.2%)	318 (83.7%)	163 (85.3%)	
	>11000	90 (15.8%)	62 (16.3%)	28 (14.7%)	
12. Serum Alkaline phosphatase (IU/L) (n=507)		452 (73 – 14960)	440 (73-14960)	496 (106-11550)	0.244
	≤450IU/L	271 (49.9%)	189 (51.8%)	82 (46.1%)	
	>450IU/L	272 (50.1%)	176 (48.2%)	96 (53.9%)	
13. Serum Albumin (g/dL) (n=531)		4.4 (2.0 – 6.2)	4.4 (2.1-6.2)	4.4 (2.0-5.6)	0.997
	<3.5g/dL	49 (9.2%)	29 (8.2%)	20 (11.4%)	
	≥3.5g/dL	482 (90.8%)	326 (91.8%)	156 (88.6%)	

(Continued)

TABLE 1 Continued

Clinical/Socio, demographic parameter (median with range)	Categories	Whole cohort (n=594)	Derivation cohort (n=396)	Validation cohort (n=198)	P-value*
*** Sociodemographic parameters**	**Median(range)**				
14. Distance from hospital (km) (n=537)		197 (20-2762)	177.5 (20-2005)	259(20-2762)	0.604
	≤100km	192 (35.8%)	138 (38.5%)	54 (30.2%)	
	>100km	345 (64.2%)	220 (61.5%)	125 (69.8%)	
15. Type of residence (n=537)	Urban	317 (59.0%)	208 (58.1%)	109 (60.9%)	0.535
	Rural	220 (41.0%)	150 (41.9%)	70 (39.1%)	
*** Survival outcomes**					
16. Mortality		217 (36.6%)	141 (35.6%)	76 (38.6%)	0.479
17. Median event free survival (months)		17.03 (14.8-19.2)	16.6 (13.3 – 20.1)	17.7 (14.4-20.9)	0.178
18. Median overall survival (months)		80 (estimate not reached)	Estimate not reached	55.7	0.820

*Continuous variables were reported as median with range. Median values between derivation cohort and validation cohort were compared during Mann-Whitney tests, while categorical variables between derivation cohort and validation cohort were compared using Chi-square test and similarly time to event outcomes were compared using log rank test.

and high risk categories were 26.0 months versus 18.5 months versus 11.8 months respectively, log rank p-value=0.002). Similarly, the median EFS was significantly different among the three risk categories in both derivation (log rank p-value<0.001) and whole cohorts (log rank p-value<0.001). The estimated 18-month EFS in the low, intermediate and high risk categories in the validation cohort are 74 ± 8%, 50 ± 6% and 29 ± 8% respectively. The corresponding values for the 36-month EFS in the validation cohort are 49 ± 9%, 32 ± 6% and 14 ± 6% respectively in the three risk groups. The 18-month and 36-month EFS values as estimated in the derivation and whole cohorts are shown in Table S1. The Harrell's c-indices of the risk score category for EFS in the derivation, validation and whole cohort were 0.682, 0.608 and 0.657 respectively. The timed AUC of ROC for predicting 18-month EFS in the derivation, validation and whole cohort were 0.67 (0.61-0.73), 0.67 (0.59-0.76) and 0.67 (0.62-0.72) respectively, while that of 36-month EFS in the derivation, validation and whole cohort were 0.68 (0.62-0.75), 0.66 (0.56-0.76) and 0.68 (0.63-0.73) respectively. (Table S1 and Figure 2).

Prognostic ability of the risk score category for overall survival

On application of the risk score category in the validation cohort, the median OS in the three categories was significantly different (median OS in the low risk, intermediate risk and high risk categories were 66 months versus 53.6 months versus 18.8 months, log rank p-value=0.027). (Table S1 and Figure S2). Similarly, the median OS was significantly different among the three risk categories in both the derivation (log rank p-value<0.001) and the whole cohort (log rank p-value<0.001) as well. The estimated 18-month OS in the low, intermediate and high risk categories in the validation cohort are 90 ± 5%, 79 ± 5% and 55 ± 9% respectively. The corresponding values for the 36-month OS in the validation cohort are 70 ± 9%, 63 ± 6% and 35± 9% respectively in the three risk groups. The 18-month and 36-month OS values in the derivation and whole cohorts are shown in Table S1. The estimated 18-month and 36-month OS in the derivation, validation and whole cohort are shown in Table S1. The Harrell's c-indices of the risk score category for OS in the derivation, validation and whole cohort were 0.681, 0.603 and 0.654 respectively. The timed AUC of ROC values for predicting 18-month OS in the derivation, validation and whole cohort were 0.68 (0.62-0.74), 0.68 (0.59-0.77) and 0.67 (0.63-0.73) respectively, while that for 36-month OS in the derivation, validation and whole cohort were 0.66 (0.60-0.73), 0.63 (0.54-0.73) and 0.66 (0.61-0.71) respectively. (Table S1 and Figure S2).

Impact of burden of metastases on survival

Among the 204 patients with metastatic disease at baseline, 143 (70.1%) had lung-only metastases, 42 (20.6%) had lung and bone metastases, 15 (7.4%) patients had isolated bone metastases and 4 (2%) had other sites of metastases. In the metastatic cohort, 56 patients (27.5%) had limited burden metastases while 148 patients (72.5%) had extensive metastases. It was seen that EFS in patients with limited burden metastases was significantly better than that of patients with extensive metastases (HR 0.62; p=0.007) but worse than that of patients with localised disease. (HR 2.2; p<0.001). However, OS of the cohort with limited metastatic burden was similar to patients with localised disease (HR 1.39; p=0.183) (Figure S3). Metastasectomy of lung metastases was done in 10 (5.15%) of 194 patients in the upfront setting.

In our patient cohort, 198 patients progressed after first line therapy. This included 23(11.6%) local-only recurrences, 118 (59.6%) isolated lung metastases, 40 (20.2%) patients with lung and local site recurrences, 8 patients (4.04%) with isolated bone metastases and 32 (16.2%) patients with metastases at other/

TABLE 2 Univariable and multivariable analyses of prognostic factors for event free survival in the derivation cohort (n=396).

Prognostic factors	Categories (n)	Median event free survival (months)	Univariable analysis			Multivariable analysis*		
			HR	95% CI	P value	HR	95% CI	P-value
1. Age (years)	≤18 (225)	16.9	1	–	–	–	–	–
	>18 (171)	15.6	1.004	0.77, 1.31	0.974	–	–	–
2. Sex	Male (270)	16.1	1.24	0.93, 1.66	0.144	–	–	–
	Female (126)	19.6	1	–	–	–	–	–
3. Tumor diameter of primary site (Longest dimension)	≤10cm (191)	24.4	1	–	–	1	–	–
	>10cm (131)	14.9	1.68	1.25, 2.25	0.00049	1.38	1.01, 1.89	0.045
4. Site	Appendicular (331)	19.6	1	–	–	–	–	–
	Axial (20)	11.8	1.19	0.63, 2.27	0.578	–	–	–
5. Neurovascular involvement	Yes (67)	8.2	2.84	2.11, 3.81	<0.0001	–	–	–
	No (323)	22.5	1	–	–	–	–	–
6. Symptom duration	≤4 months (190)	18.1	1.26	0.94, 1.70	0.122	–	–	–
	>4 months (142)	24.4	1	–	–	–	–	–
7. Fever at baseline	Yes (34)	16.1	1.13	0.74, 1.74	0.575	–	–	–
	No (362)	16.9	1	–	–	–	–	–
8. Pathological fracture at baseline	Yes (85)	9.7	2.02	1.52, 2.69	<0.0001	–	–	–
	No (308)	21.4	1	–	–	–	–	–
9. Metastases at baseline	Yes (131)	8.3	3.39	2.60, 4.43	<0.0001	3.55	2.58, 4.88	<0.0001
	No (265)	37.5	1	–	–	1	–	–
10. Hemoglobin (g/dL)	<11 (138)	13.8	1.38	1.05, 1.81	0.021	–	–	–
	≥11 (244)	18.3	1	–	–	–	–	–
11. Total leucocyte count (/μL)	≤11000 (318)	17.8	1	–	–	–	–	–
	>11000 (62)	14.1	1.16	0.78, 1.60	0.552	–	–	–
12. Serum Albumin (g/dL)	≥3.5 (326)	16.6	1	–	–	–	–	–
	<3.5 (29)	16.9	1.26	0.77, 2.04	0.352	–	–	–
13. Serum Alkaline Phosphatase (IU/L)	≤450 (189)	26.3	1	–	–	1	–	–
	>450 (176)	13.6	1.57	1.19, 2.08	0.0014	1.50	1.10, 2.05	0.010

HR, Hazard Ratio; CI, Confidence interval; Hazard of reference category is represented as 1.
*Multivariable analysis was done including variables with p ≤ 0.1 in univariable analyses in a forward stepwise manner based on likelihood ratio and only significant variables (p<0.05) in the multivariable model was reported.

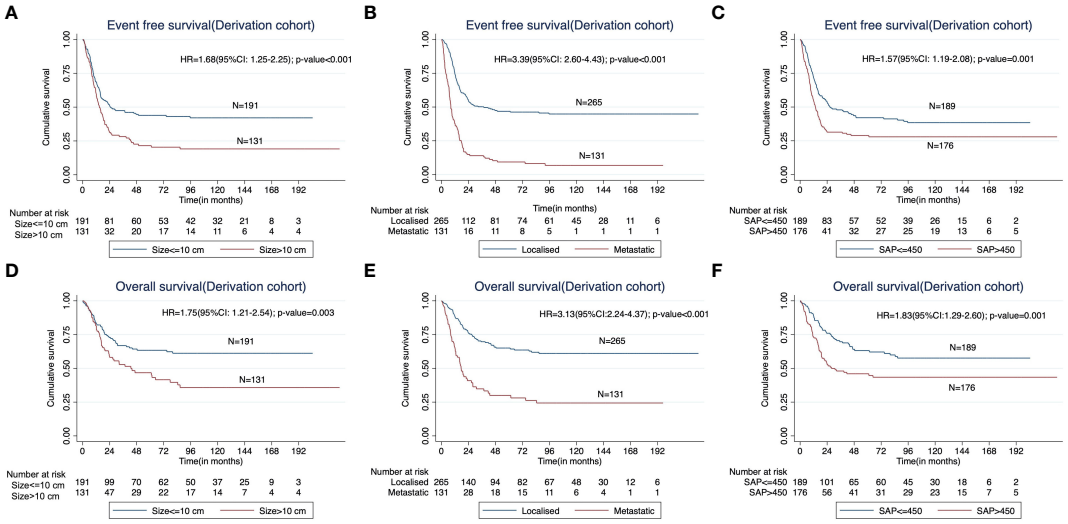

FIGURE 1
Kaplan Meier curves showing impact of **(A)** the baseline tumor size (< 10 cm versus > 10 cm), **(B)** presence of metastases at presentation, and **(C)** higher baseline serum alkaline phosphatase (≤450IU/L vs >450IU/L) on the event free survival (EFS) in the derivation cohort. The impact of the corresponding factors on overall survival (OS) is shown in **(D–F)**.

multiple sites. Among the 161 patients having lung metastases at first relapse, metastasectomy was done for 48 patients (29.81%).

Sociodemographic factors and their impact on baseline clinical factors and survival outcomes

In this study, the patients predominantly hailed from an urban residence (317/594; 59.0%) with median distance from the hospital of 197 km (20 to 2762 km), with similar distribution in the derivation and validation cohorts. The impact of residence and distance from the hospital on baseline clinical factors and survival outcomes is summarized in Table 3. The primary residence of the patient and the distance of the residence from the hospital were not predictive of either EFS or OS in the whole cohort. However, on multivariable analysis, patients with primary urban residence were more likely to have baseline tumor size greater than 10 cm (48.1% vs 37.5%, multivariable odds ratio 1.69; 95% CI: 1.13, 2.53, p=0.011) and less likely to have elevated total leucocyte count of more than 11000/μL (13.1% vs 20.0%; multivariable odds ratio 0.54; 95% CI: 0.31, 0.92; p=0.023). None of the remaining tumor characteristics or laboratory parameters significantly differed based on the type of primary residence or distance from the hospital (Table 3).

Discussion

In this study, we analysed a retrospective cohort of osteosarcoma patients treated at our centre using a uniform non-HDMTX-based protocol. We formulated and validated a prognostic score based on baseline clinical factors and tailored to a unique population of patients treated in a resource constrained setting with a non-HDMTX-based protocol. Our survival outcomes were similar to those reported in smaller studies from LMICs but still lags behind those reported from Western countries (8, 20, 31, 32).

We identified metastases, tumour size and serum alkaline phosphatase to be important determinants of survival. The presence of metastases is a universally established prognostic factor in osteosarcoma (33). We observed that patients with limited burden metastatic disease had better EFS than those with extensive burden metastatic disease. It has been previously observed that osteosarcoma presenting only with lung metastases has better survival outcome than metastases at other sites (34). However, in our cohort, the proportion of patients ultimately undergoing metastasectomy remained low compared to eligible patients, which may be partially owing to resource limitations inherent to an LMIC setting. This exemplifies the need for better interdisciplinary coordination for implementing uniform protocols for metastasectomy for patients with limited number of lung metastases.

Large size and elevated alkaline phosphatase are surrogate markers for tumour burden. Large tumour size may hinder the penetration of drugs, thereby reducing chemosensitivity. Consequently, it has been identified to be prognostic for response to therapy and survival in prior studies (8, 35). Serum alkaline phosphatase is an indicator of osteoblastic activity and thus, may be indicative of disease aggressiveness (36). The normalisation of alkaline phosphatase following completion of neoadjuvant therapy has been identified to be a predictor of better survival; however, this was not assessed in the current study (37). Biomarkers of a systemic pro-inflammatory state such as total leukocyte count and hypoalbuminemia in Ewing sarcoma and hypoalbuminemia in both Ewing and soft tissue sarcomas have been seen to have prognostic value (38–40). However, these factors do not appear to be major predictors of treatment outcomes in osteosarcoma. The

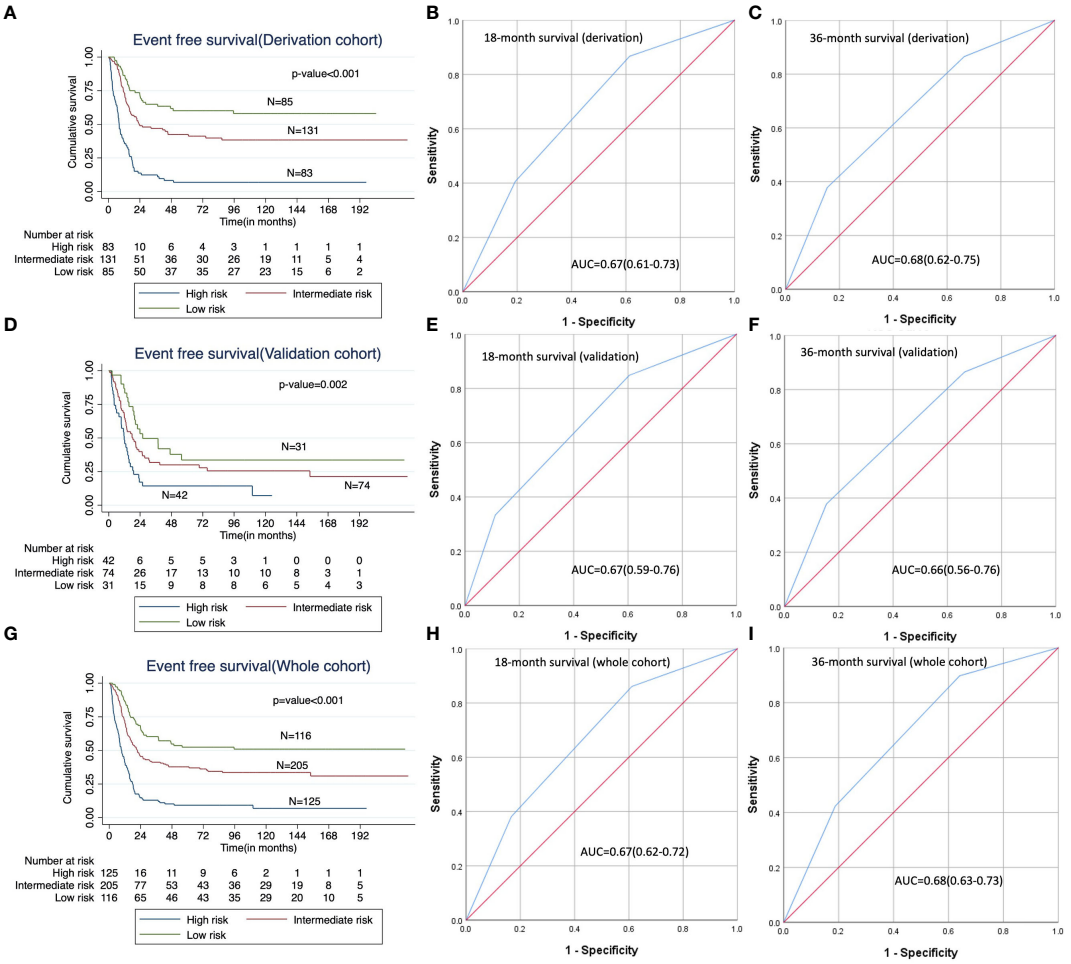

FIGURE 2
Predictive ability of the risk score category; **(A, D, G)**: Kaplan Meier curves showing impact of risk score category on EFS in the derivation, validation and whole cohorts respectively; **(B, E, H)**: Receiver operating characteristic (ROC) curves for the risk score categories for 18-month EFS in the derivation, validation and whole cohorts respectively; **(C, F, I)**: Receiver operating characteristic (ROC) curves for the risk score categories for 36-month EFS in the derivation, validation and whole cohorts respectively.

difference may be a consequence of differences in tumour microenvironmental profiles in the two tumours (41).

The prognostic factors identified in our cohort were largely similar to those described in HDMTX-based protocols. There are only few retrospective studies assessing prognostic factors while using non-HDMTX-based regimens in LMICs (31, 32, 42, 43). An analysis of another patient cohort from India using the non-HDMTX-based OGS-12 protocol has described serum alkaline phosphatase as prognostic for survival (43). Histologic response to chemotherapy has been described to be predictive in the studies available from LMICs (32, 42, 43). Metastases at presentation, tumour site and type of surgery were additionally identified to be prognostic in a Brazilian treatment cohort (44). The smaller size of the cohorts described, the shorter durations of follow up and the incorporation of treatment-related factors makes it difficult to generalise their results. Multicentre collaborative individual patient level data compilation may further our understanding of osteosarcoma in LMICs.

We designed a disease risk score based on the prognostic factors identified which had good discriminative value for distinguishing between groups with different survival. The tools currently available for risk stratification in osteosarcoma are derived predominantly from registry databases, which are inherently heterogenous in terms of institutional practices and regimens used (45–48). Although data derived from major randomised trials has enriched our understanding of prognostic factors in osteosarcoma, treatment in the setting of a trial may be subject to bias introduced by patient selection and differences in patient care as compared to real world data, thus making extrapolation difficult (8, 20, 49). Most scores have incorporated treatment-related factors into their algorithm (46, 47, 50, 51). Since treatment decisions may be altered based on baseline characteristics, such scores may be difficult to interpret. Our score was derived from a uniform single institution cohort using only basic clinical and lab parameters at presentation to allow for better risk stratification and prognostication at baseline. Furthermore, it is the only score available that is uniquely tailored to the LMIC setting accounting for treatment constraints and social backgrounds.

In current practice, non-HDMTX-based protocols incorporating risk stratified therapy, risk assessment is based on

TABLE 3 Impact of sociodemographic parameters on clinical factors at presentation and survival outcomes of osteosarcoma in the whole cohort.

Parameter	Categories	Type of primary residence (n=537)			Distance of residence from hospital (n=537)		
		Urban residence (n=317)	Rural residence (n=220)	P value	≤100 km (n=192)	>100km (n=345)	P value
1. Age (years)	≤18	197 (62.1%)	133 (60.5%)	0.692	120 (62.5%)	210 (60.9%)	0.710
	>18	120 (37.9%)	87 (39.5%)		72 (37.5%)	135 (39.1%)	
2. Sex	Male	217 (68.5%)	157 (71.4%)	0.471	128 (66.7%)	246 (71.3%)	0.263
	Female	100 (31.5%)	63 (28.6%)		64 (33.3%)	99 (28.7%)	
3. Tumor size at primary site (longest dimension)	≤10cm	135 (51.9%)	110 (62.5%)	0.029	92 (58.2%)	153 (55.0%)	0.518
	>10cm	125 (48.1%)	66 (37.5%)		66 (41.8%)	125 (45.0%)	
4. Site of primary tumor	Axial	14 (5.0%)	14 (7.0%)	0.364	9 (5.5%)	19 (6.0%)	0.804
	Appendicular	266 (95.0%)	187 (93.0%)		156 (94.5%)	297 (94.0%)	
5. Symptom duration	≤4 months	150 (56.8%)	110 (59.8%)	0.532	104 (63.8%)	156 (54.7%)	0.061
	>4 months	114 (43.2%)	74 (40.2%)		59 (36.2%)	129 (45.3%)	
6. Neurovascular bundle involvement	Yes	59 (18.9%)	33 (15.3%)	0.290	37 (19.9%)	55 (16.1%)	0.277
	No	253 (81.1%)	182 (84.7%)		149 (80.1%)	286 (83.9%)	
7. Fever at baseline	Yes	33 (10.4%)	22 (10.0%)	0.878	20 (10.4%)	35 (10.1%)	0.921
	No	284 (89.6%)	198 (90.0%)		172 (89.6%)	310 (89.9%)	
8. Pathological fracture at baseline	Yes	64 (20.4%)	40 (18.3%)	0.544	42 (22.1%)	62 (18.1%)	0.261
	No	250 (79.6%)	179 (81.7%)		148 (77.9%)	281 (81.9%)	
9. Metastases at baseline	Yes	110 (34.7%)	75 (34.1%)	0.884	67 (34.9%)	118 (34.2%)	0.871
	No	207 (65.3%)	145 (65.9%)		125 (65.1%)	227 (65.8%)	
10. Hemoglobin (g/dL)	<11	111 (36.3%)	80 (38.1%)	0.674	65 (35.1%)	126 (38.1%)	0.508
	≥11	195 (63.7%)	130 (61.9%)		120 (64.9%)	205 (61.9%)	
11. Total leucocyte count (/μL)	≤11000	265 (86.9%)	168 (80.0%)	0.036	158 (85.9%)	275 (83.1%)	0.407
	>11000	40 (13.1%)	42 (20.0%)		26 (14.1%)	56 (16.9%)	
12. Serum Albumin (g/dL)	<3.5	28 (10.1%)	19 (9.5%)	0.840	18 (10.5%)	29 (9.5%)	0.721
	≥3.5	249 (89.9%)	180 (90.5%)		153 (89.5%)	276 (90.5%)	
13. Serum Alkaline phosphatase (IU/L)	≤450	133 (46.8%)	109 (53.2%)	0.166	87 (49.4%)	155 (49.5%)	0.985
	>450	151 (53.2%)	96 (46.8%)		89 (50.6%)	158 (50.5%)	
14. Median event free survival (months)		19.1 (15.5, 22.7)	16.9 (12.1, 21.7)	0.987	19.6 (15.5, 23.8)	17.0 (13.2, 20.7)	0.914
15. Median overall survival (months)		Estimate not reached	64.7 (Estimate not reached)	0.359	59.4 (Estimate not reached)	Estimate not reached	0.556

neoadjuvant chemotherapy response. Thus, treatment escalation for high risk disease has only been practised at completion of neoadjuvant chemotherapy (30, 52). The identification of high risk patients based on baseline characteristics may allow us to better demarcate candidates for upfront intensified therapy. The use of multiple non-cross-resistant drugs at therapy initiation may allow for better tumour responses in the context of high risk disease (53). The outcomes observed in patients with metastatic disease of high risk disease based on the score formulated are demonstrably poor. Thus, the score may be used to demarcate a subset of patients who may benefit from a palliative approach with early treatment de-intensification to avoid therapy-related and consequent reductions in quality of life (54, 55).

Social barriers to healthcare accessibility may lead to delays in treatment-seeking and may adversely affect compliance. We observed that patients with urban residence were more likely to present with larger sized tumors with lower total leukocyte counts; however, it did not have any impact on survival outcomes. A study from a Western country observed that residing at greater distances from the treatment centre and in areas of high unemployment was

associated with higher mortality rates among osteosarcoma patients (56). Although social factors were integrated into our model, they did not have any significant impact on survival outcomes. This is further affirmed by our prior observation in bone sarcomas, where even in the context of resource challenged settings, tumour biology is a stronger determinant of the diagnostic interval than social factors (57). In addition, it has been seen that therapy-related factors such as delay in time to surgery following neoadjuvant chemotherapy and delay in the completion of planned therapy may compromise treatment outcomes (58, 59). Thus, optimising the delivery of healthcare services may allow for further improvements in survival.

The study represents the largest single institutional dataset of patients treated with a uniform non-HDMTX-based protocol. Furthermore, it is the largest dataset derived from a single institutional cohort in Asia. It provides a tool that allows the clinician to use baseline clinical and laboratory characteristics for risk stratification. It integrates social factors with clinical characteristics to better characterise the disease from the perspective of a resource-challenged setting. However, our study has a few limitations. Compliance to treatment and socioeconomic status were not assessed separately; thereby, their roles as potential prognostic factors could not be studied. However, the social background provided by the place of residence and distance from the treating centre may possibly serve as their surrogates. In the future, prospective studies may be formulated that evaluate the role of risk stratified therapy based on baseline characteristics to further improve outcomes.

Conclusion

This study describes a large single institutional series of patients with osteosarcoma from an LMIC treated with a uniform non-HDMTX-based protocol. Clinical factors prognostic for survival at baseline were identified and used to derive and validate a risk score for prognostication. Tumour biologic characteristics were found to supersede social factors as determinants of survival.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Institute Ethics Committee, All India Institute of Medical Sciences, New Delhi, India. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

SG analysed data, interpreted results, and wrote the manuscript. AS conceptualized the study, compiled the data, interpreted results, and wrote the manuscript. DP, SK, VK, LK, MS, AM, AB and ST conceptualized the study, provided intellectual inputs, administrative support and edited the manuscript. SB conceptualized the study, provided administrative support, intellectual inputs, interpreted results, wrote, and edited the manuscript. All authors have reviewed and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1148480/full#supplementary-material

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Similar additive effects of doxorubicin in combination with photon or proton irradiation in soft tissue sarcoma models

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High-precision radiotherapy with proton beams is frequently used in the management of aggressive soft tissue sarcoma (STS) and is often combined with doxorubicin (Dox), the first-line chemotherapy for STS. However, current treatment approaches continue to result in high local recurrence rates often occurring within the treatment field. This strongly indicates the need of optimized treatment protocols taking the vast heterogeneity of STS into account, thereby fostering personalized treatment approaches. Here, we used preclinical STS models to investigate the radiation response following photon (X) or proton (H) irradiation alone and in combination with different treatment schedules of Dox. As preclinical models, fibrosarcoma (HT-1080), undifferentiated pleiomorphic sarcoma (GCT), and embryonal rhabdomyosarcoma (RD) cell lines were used; the latter two are mutated for TP53. The cellular response regarding clonogenic survival, apoptosis, cell-cycle distribution, proliferation, viability, morphology, and motility was investigated. The different STS cell types revealed a dose-dependent radiation response with reduced survival, proliferation, viability, and motility whereas G2/M phase arrest as well as apoptosis were induced. RD cells showed the most radiosensitive phenotype; the linear quadratic model fit could not be applied. In combined treatment schedules, Dox showed the highest efficiency when applied after or before and after radiation; Dox treatment only before radiation was less efficient. GCT cells were the most chemoresistant cell line in this study most probably due to their TP53 mutation status. Interestingly, similar additive effects could be observed for X or H irradiation in combination with Dox treatment. However, the additive effects were determined more frequently for X than for H irradiation. Thus, further investigations are needed to specify alternative drug therapies that display superior efficacy when combined with H therapy.

KEYWORDS

soft tissue sarcoma, proton beam radiotherapy, combined treatment, doxorubicin, additive effect

1 Introduction

Sarcomas are a very rare disease with an incidence of 6 per 100.000 people representing 1%–2% of all adult and 12%–15% of all pediatric cancers (1). They originate from soft (mesenchymal) tissues (84%) or bones (14%) (2). Classification, including immunohistochemistry, is important in the context of diagnosis and therapeutic option (3–5). Currently, >70 histological subtypes with specific morphology have been identified so far (3). Rhabdomyosarcomas (RMS) are the most common soft tissue tumor (STS) in children accounting for >50% of the cases (6). Undifferentiated pleiomorphic sarcoma (UPS) including giant cell tumors (GCT) is the most common STS in late adulthood with a high rate of local recurrence and distal metastasis (7) and 5-year survival of patients of ca. 50% (4). Fibrosarcoma generally concerns all age groups, but subtypes vary significantly between adults and children, e.g., rarely metastasizing infantile to highly malignant adult-type fibrosarcoma with poor prognosis (7). Independent of histology, sarcomas are generally treated multimodally in expert reference centers since there is a high need for individualized treatment approaches (8). Whereas surgical resection of the tumor remains as a primary treatment option, high-precision neoadjuvant or adjuvant radiotherapy (RT) was shown to improve local control rates (9). In particular, proton beam therapy (PBT) is gaining importance as a treatment option for STS due to the advantageous dose distribution. In contrast to photon-based intensity-modulated radiotherapy, PBT can spare critical normal tissue structures such as the central nervous system or other organs better while delivering an iso-effective dose to the tumor volume (2). The effects of photon (X) and proton (H) beams can be compared for various biological endpoints via the relative biological effectiveness (RBE). The RBE sets the photon and H doses, which induce the same biological effect in relation. In clinical treatment planning, the RBE of H is considered to be a constant 1.1 (10). In contrast, a large heterogeneity in RBE of H was shown for various sarcoma cell lines *in vitro* (11). STS shows a poor response to systematic treatments (9), and first-line drugs are still classical chemotherapies such as doxorubicin (Dox, anthracycline), ifosfamide, and dacarbazine (both alkylating drugs). The survival benefit for STS patients with low predicted overall survival was confirmed for anthracycline-based chemotherapy (12). However, alternative regimes to improve outcomes of STS such as combined radiation and chemotherapy approaches remain challenging (13). Despite recent advances in newly approved drugs and radiotherapy modalities, the 5-year overall survival for large and high-grade tumors is still poor with rates below 50% (14). Thus, there is an urgent need to optimize treatment protocols for combined radiochemotherapies, particularly with PBT and standard chemotherapy in STS (11), and to investigate (potential) additive effects of combined therapies relative to the mono-radiotherapy (15). This study therefore characterizes the effects of H irradiation alone and compares the effect to X irradiations alone and in combination with Dox in preclinical STS models (fibrosarcoma, undifferentiated pleiomorphic sarcoma, rhabdomyosarcoma). Furthermore, the sequence of the combined treatments was altered by applying Dox only before, before and after, or only after irradiation to gain insights in the effect size of chemotherapy and radiation modalities.

2 Materials and methods

2.1 Cell culture

The HT-1080 (ATCC CCL-121, fibrosarcoma, RD (ATCC CCL-136, embryonal rhabdomyosarcoma), and GCT (ATCC TIB-233, undifferentiated pleomorphic sarcoma/giant cell tumor) cell lines were obtained from the American Type Culture Collection. HT-1080 cells were isolated from a 35-year-old man who did not receive treatment. The cells are TP53 wild type (16). RD cells were derived from biopsy specimens of a 7-year-old woman with pelvic RMS previously treated with cyclophosphamide and radiation. GCT cells were derived from the lung of a 29-year-old man. The TP53 gene was mutated in RD (homozygous (17) and GCT (two heterozygous) cells. All cell lines were grown in medium supplemented with 10% (v/v) fetal bovine serum and penicillin–streptomycin (100 U/ml). The HT-1080 and RD cell lines were grown in Dulbecco's modified Eagle's medium (Thermo Fisher scientific, Waltham, USA), which was supplemented with 1% sodium pyruvate (Sigma-Aldrich, St. Louis, USA) for HT-1080 cells. GCT cells were grown in McCoy's (Thermo Fisher Scientific, Waltham, USA). Cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

2.2 Photon irradiation

Photon irradiation hereafter referred to as X was performed using an ISOVOLT 320 X-ray machine (Seifert–Pantak, East Haven, CT) at 320 kV, 10 mA with a 1.65-mm aluminum filter, and a distance around 50 cm to the object being irradiated (18).

2.3 Proton irradiation

Proton irradiation hereafter referred to as H was performed with an IBA Proteus PLUS proton therapy system (IBA PT, Louvain-la-Neuve, Belgium) at the West German Proton Therapy Centre Essen (WPE). A clinical pencil beam scanning line with an IBA PBS-dedicated nozzle was used. Several proton beams were energy and intensity modulated layered to form a spread-out Bragg peak (SOBP) consisting of five energy layers of 118.8 MeV up to 129.9 MeV. The proton beam range was compensated with a range shifting block water equivalent thickness (WET) = 74 mm, material: polymethyl methacrylate (PMMA)) and an additional solid water phantom (RW3 plates, type SP34 IBA Dosimetry, composition: 98% polystyrene + 2% TiO_2) with a WET of 3.3 cm to irradiate the cells in the middle of the SOBP. Cells in multiwell plates were irradiated with a homogeneous field with absorbed physical doses of 1, 2, 4, 6, or 8 Gy (field sizes: $20 \times 20 \times 1$ cm^3). Multiwell plates were positioned laterally and centered with the sample surface in the

isocenter on the treatment table and irradiated with a gantry angle of 0°.

2.4 Doxorubicin treatment

The cytotoxic antibiotic doxorubicin (Dox) (2 mg/ml, Medac GmbH, Wedel, Germany) was purchased from and prepared by the pharmacy of the University Hospital Essen. For experiments, Dox was diluted in PBS (Invitrogen, Carlsbad, USA) and culture medium. Cells were treated in different sequences: 3 h before irradiation (DoxA), 3 h before irradiation and refreshed within 1 h after irradiation till the end of the experiment (DoxB), or 1 h after irradiation till the end of the experiment (DoxC) (Figure 1D).

2.5 Conditioned media

RD or GCT cells were cultured in normal growth media until confluence. The medium was collected, centrifuged, sterile-filtered (0.2 µm, Roth, Karlsruhe, Germany), and stored at −20°C until use. The conditioned medium was mixed with fresh medium as a 20% mixture for RD cells and a 40% mixture for GCT cells for the colony formation assay (19).

2.6 Colony formation assay

For the clonogenic survival, HT-1080 and GCT cells were preseeded 8 h and RD cells 24 h prior to radiation in triplicates in six-well plates. Cells were treated with Dox-containing culture medium. Following the irradiation, the media of all samples were changed with medium (HT-1080), conditioned medium (GCT and RD), or Dox-containing (conditioned) medium. The colonies were fixed after 9 (HT-1080), 10 (GCT), or 12 (RD) days depending on the cell doubling time (HT-1080: 24 h, GCT: 26 h, RD: 48 h), stained using 0.3% crystal violet dye (Roth, Karlsruhe, Germany) in 70% ethanol for 10 min at RT, rinsed with water, and air dried. Colonies with 50 cells were scored as surviving.

2.7 Flow cytometry analysis

Cells were plated 24 h before treatment in six-well plates. Propidium iodide (PI) staining and flow cytometry analysis for apoptotic DNA fragmentation (subG1 population) were performed 48, 72, or 96 h post treatment. Cells were incubated for 15–30 min at RT with a staining solution (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, 0.05%, Triton X-100 (all Roth, Karlsruhe, Germany)), additional 62 µg/ml RNase A (AppliChem, Darmstadt, Germany), and 40 µg/ml PI (Sigma-Aldrich, St. Louis, USA) (20). Samples were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany; FL-2) as described elsewhere (18). Cell-cycle phase distribution was analyzed with Kaluza software to identify the subG1 population (apoptotic DNA fragmentation, whole population), and in a second step, the living cell population (G1, S, G2/M phase) was investigated for a G2 arrest. Statistical analysis was performed in GraphPad Prism Version 8.3.0.

2.8 Migration assay

The migratory potential of cells was investigated with the migration assay 48 h post treatment at 0, 3, 6, 9, 24, and 48 h time points after scratch induction (Supplementary Figure 1). Wound closure was documented in images and determined by measuring the area of the scratch using ImageJ (Wayne Rasband, National Institutes of Health, US states) with the plugin Wound_healing_size_tool_updated (19). To calculate the maximum motility speed for each cell line, we calculated a simple linear regression between two time points (HT-1080: 0–3 h, GCT: 3–6 h, RD: 6–9 h) and determined the slope in the steep part of the curve. Additional morphological changes were evaluated by a sarcoma specialist on the basis of images of the migration assay.

2.9 Cell viability and proliferation analyses

The cell proliferation reagent WST-1 (in PBS 1:3, Roche, Rotkreuz, Schweiz) was used as a colorimetric assay for the quantification of cellular viability and cytotoxicity according to the manufacturer's instruction (Roche, Rotkreuz, Schweiz). Optical densities were measured at 450 nm 60–90 min after incubation (BioTek Synergy H1 microplate reader, Agilent Technologies, Santa Clara, USA). Afterward, cells were fixed with glutaraldehyde (1% in PBS, Roth, Karlsruhe, Germany) for 15 min, stained with 0.5% crystal violet (CV) dye (Roth, Karlsruhe, Germany) in deionized water for 25 min, gently rinsed in water, and air dried overnight. The crystal violet dye was resolved in ECOSURF (0.2% in PBS, Roth, Karlsruhe, Germany) on a shaker for 20 min before optical density was measured at 540 nm (19). WST-CV data were normalized to 0 Gy or 0 nM controls.

2.10 Data analysis and statistics

Cell survival and dose response data were fitted using the linear quadratic equation:

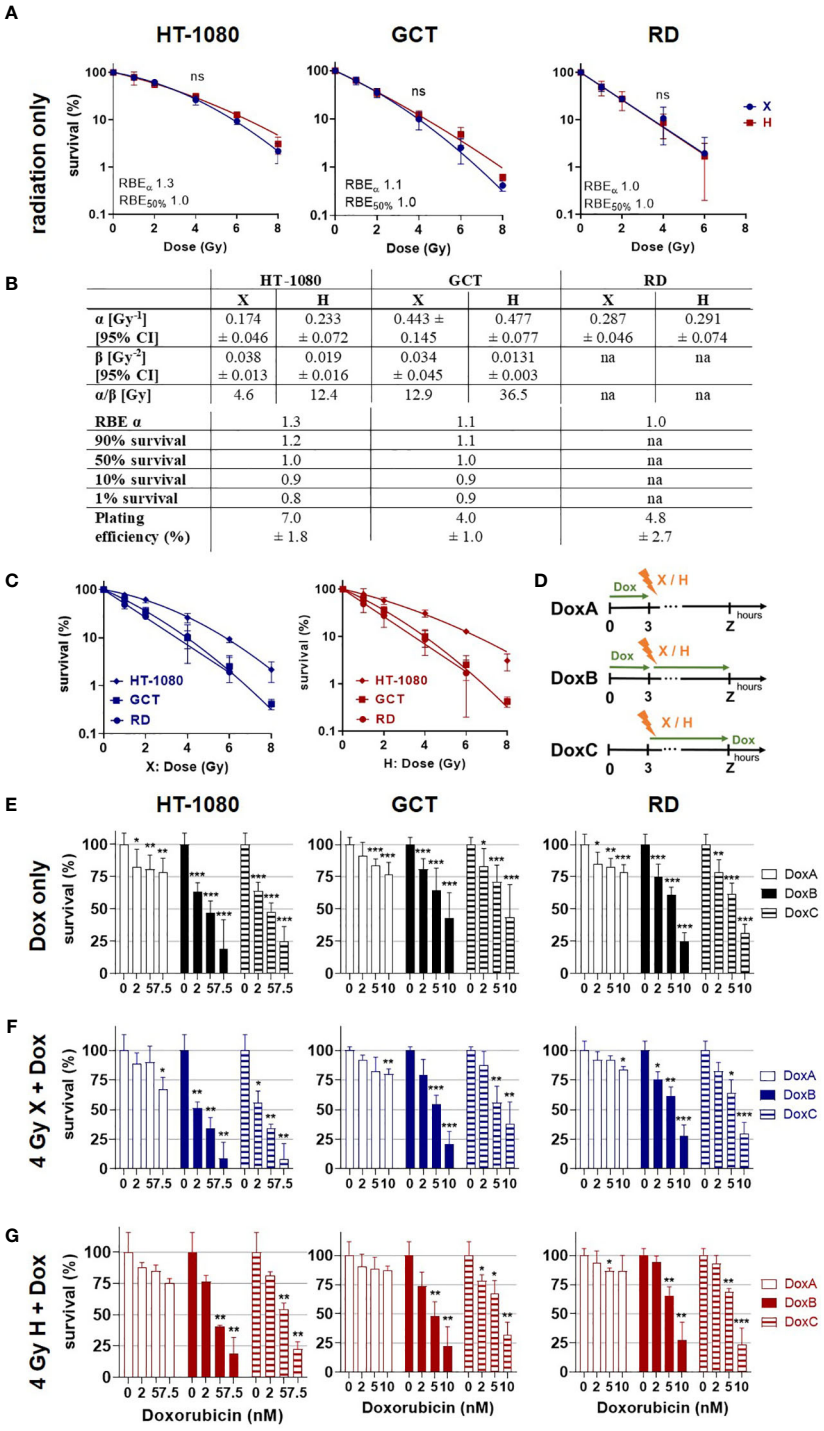
$$\mathbf{SF} = \mathbf{e}^{-(\alpha D+\beta D^2)}$$

where SF denotes the surviving fraction of cells at dose D with curve fitting parameters α and β. Non-linear regression analysis was performed on survival curves using GraphPad Prism, version 8.3.0. RBE values for protons were calculated relative to 320 kV X-rays according to

$$\mathbf{RBE\ SF} = \frac{\mathbf{D^{X\ SF}}}{\mathbf{D^{H\ SF}}}$$

where RBE SF is the RBE at a certain survival level (SF) and $D^{X\ SF}$ and $D^{H\ SF}$ are the X and H dose for an iso-effect, respectively.

Statistical analyses were performed with GraphPad Prism 8.3.0, and all data points represent at least three replicates with error bars



	HT-1080		GCT		RD	
	X	H	X	H	X	H
α [Gy^{-1}] [95% CI]	0.174 ± 0.046	0.233 ± 0.072	0.443 ± 0.145	0.477 ± 0.077	0.287 ± 0.046	0.291 ± 0.074
β [Gy^{-2}] [95% CI]	0.038 ± 0.013	0.019 ± 0.016	0.034 ± 0.045	0.0131 ± 0.003	na	na
α/β [Gy]	4.6	12.4	12.9	36.5	na	na
RBE α	1.3		1.1		1.0	
90% survival	1.2		1.1		na	
50% survival	1.0		1.0		na	
10% survival	0.9		0.9		na	
1% survival	0.8		0.9		na	
Plating efficiency (%)	7.0 ± 1.8		4.0 ± 1.0		4.8 ± 2.7	

FIGURE 1
Colony formation assay. Clonogenic survival of HT-1080, GCT, and RD cells following **(A)** X radiation (blue) or H radiation (red) alone. HT-1080/GCT: fitted with the linear–quadratic model; RD: semi log line fit. **(B)** Table summarizing the fit parameter of the survival curves shown in **(A)**, the maximum RBEα, the RBE values to survival levels 90%, 50%, 10%, and 1%, and the plating efficiency of the cell models. RD cells were fitted with a semi log line fit, and no β-term was retrieved. **(C)** Cell survival curves following X (blue) or H (red) irradiation replotted from **(A)** to allow better evaluation of the radiation quality effects. **(D)** Summary of doxorubicin (Dox) treatment schedules. DoxA: 3 h before (mock) irradiation followed by media exchange without Dox, DoxB: 3 h before (mock) irradiation followed by media exchange containing Dox. Dox exposure until end of experiment. DoxC: (mock) irradiation followed by media exchange containing Dox. Dox exposure until end of experiment. Mock Dox treatments (medium without Dox) were performed for all conditions. **(E)** Dox treatment alone or in combination with **(F)** 4 Gy X irradiation or **(G)** 4 Gy H irradiation. Dox was applied according to **(D)**. Samples were normalized to matching 0 nM (+ irradiation) controls. n ≥ 3, statistical analysis: **(A)** paired t-test for whole curve comparing X vs. H. **(E-G)** Unpaired t-test comparing mono-/combined treatment vs. matching 0 nM control. p values > 0.05 (not significant, ns), < 0.05 (*), < 0.01 (**), and < 0.001 (***) were considered statistically significant.

representing the standard deviation (SD). All presented data were normalized to the experiment, time, and treatment matching controls. The SD for the controls of each assay was calculated as followed. For the colony formation assay (CFA), the plating efficiency (PE) was calculated. The corresponding SD represents the relative mean of the PEs. For subG1 levels (apoptosis) and the cell-cycle phase, the SD was calculated from the mean of relative subG1 or cell-cycle phase levels. For the cell viability and proliferation assay, measurements were normalized to 0 Gy control and the corresponding SD was calculated from the relative mean of measurements. For the migration assay, the SD was calculated from the mean of relative motility. The significant level was determined by unpaired (curve comparison) or paired t-test (data point comparison) with p values > 0.05 (not significant, ns),< 0.05 (*),< 0.01 (**), and< 0.001 (***) were considered statistically significant.

3 Results

3.1 Clonogenic survival: combined Dox treatment reduced clonogenic survival of STS cells more efficiently upon prolonged treatment

The CFA is the most reliable method to quantify clonogenic growth and survival following radiation as an important endpoint of the cellular response toward cytotoxic stimuli (21). In order to determine the radiation sensitivity of the different STS cells, CFA was performed following X or H irradiation (dose range 0–8 Gy). Plating efficiencies and survival curves were calculated from surviving colony numbers, and respective curves were fitted with the linear quadratic model (LQM) for HT-1080 and GCT cells and with a semi-log line for RD cells (Figure 1A). RD cells seem to be the most radiosensitive cell line, followed by GCT and HT-1080. Of note, no significant difference in survival curves between X- and H-irradiated STS cells could be estimated (Figure 1A). A cell line comparison of the response to X or H irradiation showed that RD, followed by GCT and HT-1080, was the most radiosensitive cells to both radiation modalities (Figure 1C). The RBEα defined as the ratio of α_H/α_X shows for HT-1080 an elevated RBE of 1.3 indicating a higher sensitivity toward H irradiation (Figure 1B). This effect was not seen for GCT or RD cells. The RBE decreases with lower survival levels, which points toward a higher effectiveness for higher single X doses (≥6 Gy) relative to H irradiation in HT-1080 and GCT cells. Dox treatment at the indicated concentration (0–10 nM) alone was then used to determine respective chemosensitivities (Figure 1E). HT-1080 cells were most chemosensitive STS cells, and the maximum Dox concentration had to be reduced from 10 to 7.5 nM to archive surviving colonies. The longest Dox treatment, DoxB, reduced most effectively the cell survival in a dose-dependent manner (Figure 1D). In combination with X or H irradiation, the survival of all cell lines was even further reduced, again, with DoxB being most effective (Figures 1F, G). Of note, Dox treatment (only) before radiation (DoxA) was less effective than Dox after irradiation (DoxC) independent of cell model or radiation quality (Figure 1E). When comparing Dox treatment alone with combined treatment modalities, significant differences for HT-1080 (2 nM DoxB and X; 2 nM DoxB/C and H; 5 nM DoxC and X) and RD cells (2 nM DoxB/C and H) were revealed; GCT cells were not significantly affected. When comparing matching DoxA and DoxB or DoxC (alone or in combination with irradiation), significant differences for all Dox concentrations in HT-1080 and RD cells, for 5 nM and in GCT for 10 nM (Figures 1E–G) were evaluated.

3.2 Apoptosis: GCT cells are chemoresistant for Dox treatment alone independent of sequence but sensitive for combined treatment with radiation

Apoptosis is a further mechanism of cell death following radiation exposure and the main mechanism of action for the DNA damaging drug Dox (22). According to the clonogenic survival measurements performed above, apoptosis induction was analyzed next within the first 96 h following X or H irradiation and 10 nM Dox treatment by determining apoptotic DNA fragmentation using flow cytometry analysis in combination with PI staining. Relative to controls (0 Gy, 0 nM Dox), the subG1 population increased with radiation dose and time after treatment in HT-1080 and RD cells whereas in GCT cells only a radiation dose-dependent effect was seen (Figure 2A). Dox treatment alone had minor effects in HT-1080 and RD cells and did not affect GCT cells (Figure 2B). Combined X or H irradiation with Dox showed a radiation dose-dependent higher apoptosis rate and a Dox schedule-dependent difference with DoxB and DoxC being more effective than DoxA (Figures 2C–F). Matching X and H samples were compared by identifying the potential influence of the radiation quality (Supplementary Figure 2). Only the apoptosis rates in HT-1080 were statistically different following 8-Gy radiation alone (Supplementary Figure 2A). However, GCT and RD cells are shown in combination with DoxA and RD cells also in combination with DoxB significant differences following 8 Gy (Supplementary Figures 2B, C). The cellular response following DoxC was radiation quality independent (Supplementary Figure 2D). The data were normalized to the respective dose (4 or 8 Gy), radiation quality (X or H), and time matching (48, 72, 96 h) of samples to identify potential additive or synergistic effects in combined treated samples (Supplementary Figure 3). For HT-1080 cells, an additive effect could be identified for both irradiation qualities but only for DoxB and DoxC. In contrast, in GCT cells, no additional effect was seen for H irradiation and any Dox treatment. RD cells showed additive effects for X and H irradiation with DoxC (Supplementary Figure 3).

3.3 Cell cycle distribution: accumulation of the G2/M population in GCT and RD cells following treatment with irradiation or Dox

DNA damaging treatment such as radiation and chemotherapy can induce a transient or permanent cell-cycle arrest stopping the proliferation of damaged cells and providing an opportunity for

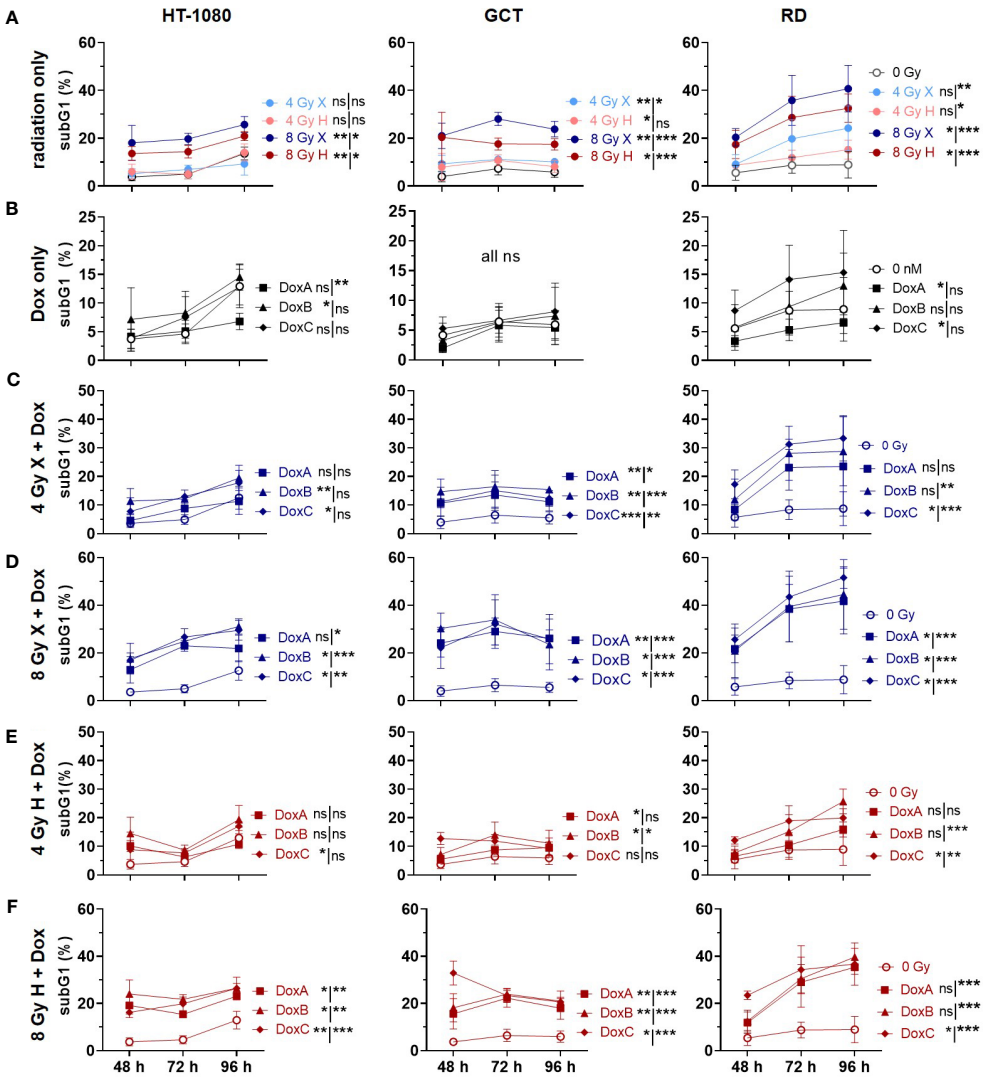


FIGURE 2
Flow cytometry comparing relative subG1 phase proportion (subG1) of whole-cell population (sub G1, G1, S, G2, M phase) of 0 Gy control and treatment of HT-1080, GCT, and RD cells following **(A)** radiation only with 4 and 8 Gy X (light/dark blue) or 4 and 8 Gy H (light/dark red). **(B)** Dox treatment with 10 nM DoxA (before), DoxB (before & after), or DoxC (after); Dox treatment schedule details in Figure 1D. **(C - F)** Combined treatment with DoxA, DoxB, or DoxC, and **(C)** 4 Gy X (blue), **(D)** 8 Gy X (blue), **(E)** 4 Gy H (red), or **(F)** 8 Gy H (red). n ≥ 3, statistical analysis: paired t-test for whole curve or unpaired t-test for 96 h timepoint (shown as whole curve | 96 h) comparing treatment vs. 0 Gy control. p values > 0.05 (not significant, ns), < 0.05 (*), < 0.01 (**), and < 0.001 (***) were considered statistically significant.

repair (23). Therefore, the effect of mono- or combined treatment with Dox and X or H radiation on cell cycle phases was analyzed (Figure 3). The HT-1080 cell did not show a cell-cycle alteration within 96 h after the indicated treatments. In contrast, GCT and RD cells accumulated in the G2/M phase 48 h after treatment with radiation only, or in combination with DoxA and DoxB. For the most intense treatment (DoxB 8 Gy X or H), 37.7% and 40.2% for GCT and 48.6% and 47.3% for RD cells accumulated in the G2/M phase at 48 h, respectively. Arrests were beginning to resolve at 96 h post treatment; significant changes relative to controls could still be detected (Figure 3).

3.4 Proliferation: prolonged Dox treatment combined with irradiation reduced proliferation activity of STS cells

Due to the G2/M phase arrest in two cell lines (GCT and RD cells), we hypothesized that radiation might also reduce the general proliferation activity. Cellular proliferation levels following irradiation and Dox treatment were then estimated for the different STS cells using the crystal violet assay (Figure 4). Relative to controls (0 Gy, 0 nM Dox), all cell lines showed reduced proliferation activities following both radiation qualities

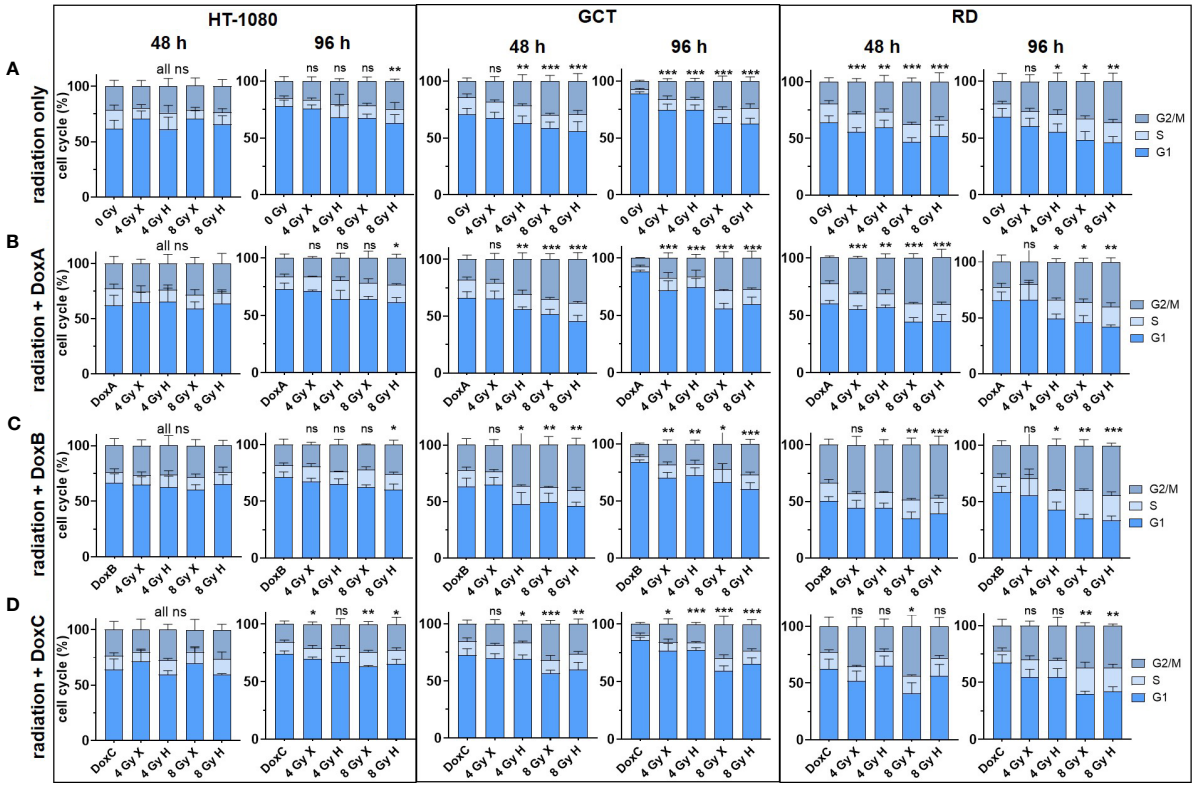


FIGURE 3
Flow cytometry comparing the relative cell-cycle phase (G1 + S + G2/M = 100%) of HT-1080, GCT, and RD cells at 48 and 96 h following **(A)** 4 and 8 Gy X or H radiation only. **(B–D)** Combined treatment with radiation and **(B)** DoxA (before), **(C)** DoxB (before and after), and **(D)** DoxC (after). Dox treatment schedule details in Figure 1D. n ≥ 3, statistical analysis: unpaired t-test for each timepoint comparing treatment vs. matching control of the G2 phase. p values > 0.05 (not significant, ns), < 0.05 (*), < 0.01 (**), and < 0.001 (***) were considered statistically significant.

in a dose-dependent manner (Figure 4A). Dox treatment alone—in either treatment schedule—exhibited effects on the proliferation levels of GCT cells. DoxB and DoxC treatment schedules in contrast were able to reduce proliferations in HT1080 and RD cells (Figure 4B). When using radiation treatment in addition, all combinatory treatments significantly lowered proliferation activities of all STS cell lines investigated 96 h post onset of treatment (Figures 4C-F). Time- and dose-matching X- and H-exposed samples were additionally compared by identifying the potential influence of the radiation quality (Supplementary Figure 4). No significant changes could be found with the exemption of whole curve comparison of HT-1018 cells following 4 Gy and DoxA (Supplementary Figure 4B). To identify potential additive or synergistic effects in combined treated samples, the data were normalized to the respective dose (4 or 8 Gy), radiation quality (X or H), and time matching (48, 72, or 96 h) samples (Supplementary Figure 5). GCT cells were the most affected cell line, and additive effects were found for all Dox conditions with X irradiation (Supplementary Figures 5A, B). Following H irradiation, much fewer effects could be detected. In contrast, RD cells were the least affected cell line (Supplementary Figures 5B, C). However, DoxB seems to be the most efficient for all cell lines (Supplementary Figures 5).

3.5 Cell viability: additive effects could be identified for prolonged Dox treatment and X but not for H

Cellular viabilities were measured via metabolic activities following combined treatment of X or H irradiation with Dox using the WST-1 reagent (Figure 5). Relative to controls (0 Gy, 0 nM Dox), both radiation qualities lowered the cell viability in a dose-dependent manner. GCT recovered independent of radiation quality to the control level after 4 Gy and 96 h, whereas HT-1080 and RD did not (Figure 5A). Dox alone had only minor effects on cellular viabilities; GCT cells were not affected, whereas minor effects of DoxB (HT-1080 cells) and DoxC (HT-1080, RD cells) were seen (Figure 5B). The combination of X and all Dox treatments significantly reduced the cell viability in HT-1080 and RD cells 96 h post treatment; in GCT cells, only 8 Gy X and DoxA and DoxB was effective (Figures 5C-D). To 96 h post treatment, H and Dox significantly decreased metabolic activity in all cell lines and treatments except HT-1080 to 4 Gy DoxB and DoxC (Figures 5E-F). Time- and dose-matching X- and H-irradiated samples were assessed to identify the potential influence of the radiation quality (Supplementary Figure 6). Again, no significant difference between X- and H-irradiated samples 96 h post treatment

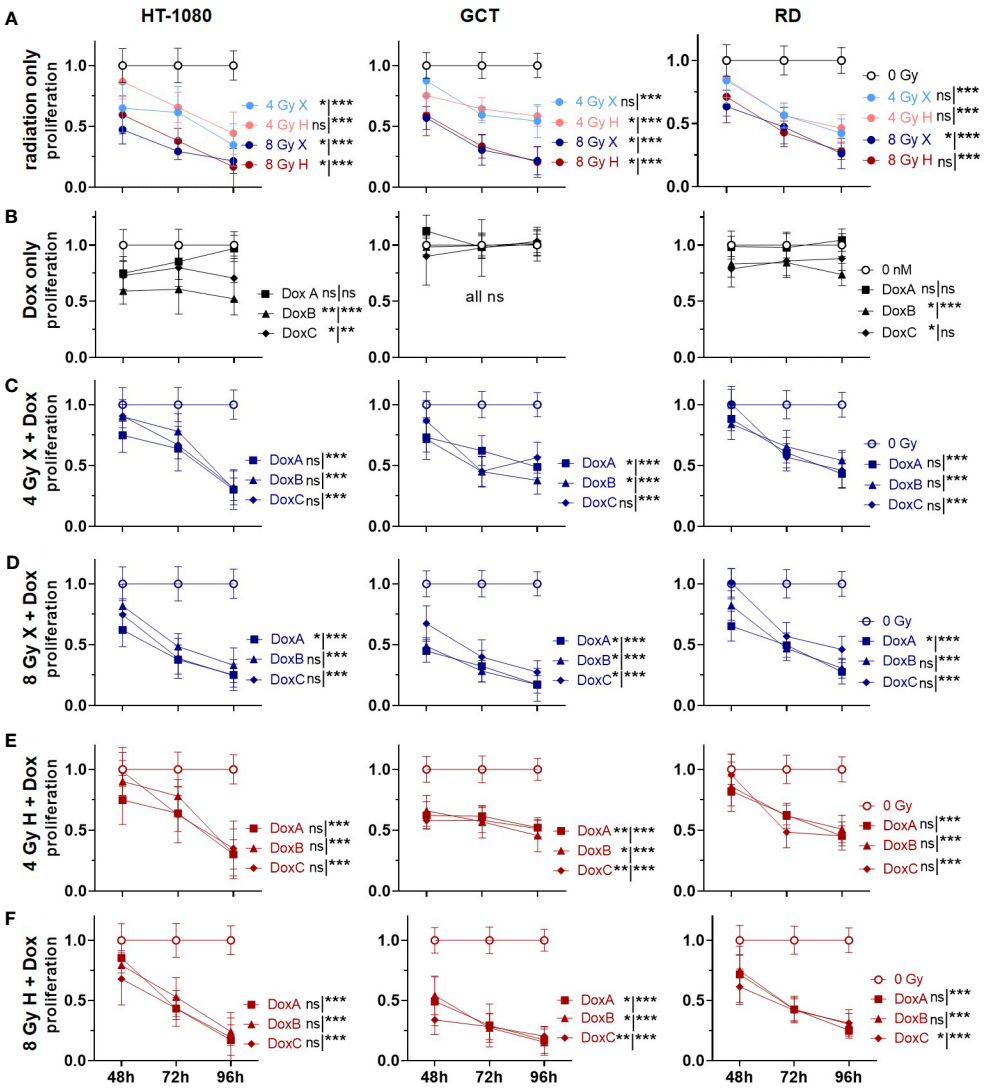


FIGURE 4
CV assay comparing the relative number of proliferating cells (proliferation; normalized to matched control) of 0 Gy control vs. treatment of HT-1080, GCT, and RD cells following **(A)** radiation only with 4 and 8 Gy X (light/dark blue) or 4 and 8 Gy H radiation (light/dark red). **(B)** Dox treatment with 10 nM DoxA (before), DoxB (before and after), or DoxC (after). Dox treatment schedule details in Figure 1D **(C–F)** Combined treatment with DoxA, DoxB, or DoxC and **(C)** 4 Gy X (blue), **(D)** 8 Gy X (blue), **(E)** 4 Gy H (red), and **(F)** 8 Gy H (red). n ≥ 3, statistical analysis: paired t-test for whole curve or unpaired t-test for the 96 h timepoint (shown as whole curve | 96 h) comparing treatment vs. 0 Gy control. p values > 0.05 (not significant, ns), < 0.05 (*), < 0.01 (**), and < 0.001 (***) were considered statistically significant.

could be found, exempt GCT to 4 Gy (96 h) or 8 Gy (whole curve). In contrast, whole curve comparisons showed significant changes for HT-1080 and RD following irradiation and DoxA or DoxB. (Supplementary Figures 6B, C). To further reveal potential additive effects in combined treated samples, data were normalized to the respective dose (4 or 8 Gy), radiation quality (X or H), and time matching (48, 72, 96 h) samples (Supplementary Figure 7). For X-exposed samples, additive effects were found for HT-1080 and RD cells whereas GCT cells were not affected (Supplementary Figures 7A, B). No additive effects were identified following H irradiation and any Dox treatment (Supplementary Figures 7C, D).

3.6 Cell morphology analysis post treatment: radiation effects morphology more pronounced than Dox

The migration assay was used to study morphological changes upon treatment (Figure 6, Supplementary Figure 1). Untreated HT-1080 cells, under the given cell culture conditions, appear small, with a spindled to round shape, with aspects of a whirling architecture. The nuclei are hyperchromatic and broadly isomorphic. Upon X and H irradiation, HT-1080 cells seem slightly enlarged and appear predominantly in spindle shape with

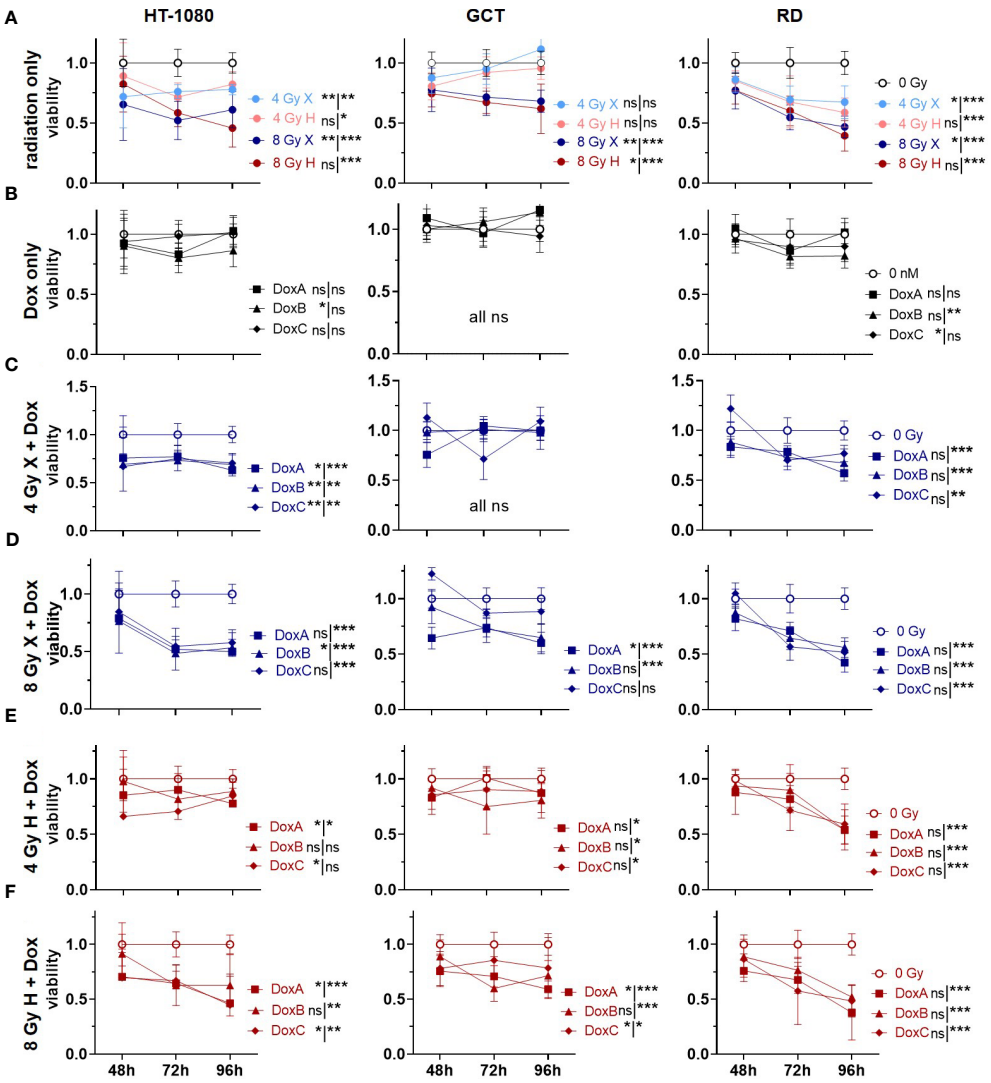


FIGURE 5
WST assay comparing relative number of viable cells (viability; normalized to matched controls) of 0 Gy control vs. treatment of HT-1080, GCT, and RD cells following **(A)** radiation only with 4 and 8 Gy X (light/dark blue) or 4 and 8 Gy H radiation (light/dark red). **(B)** Dox treatment only with 10 nM DoxA (before), DoxB (before and after), or DoxC (after). Dox treatment schedule details in Figure 1D **(C–F)** Combined treatment with DoxA, DoxB, or DoxC and **(C)** 4 Gy X (blue), **(D)** 8 Gy X (blue), **(E)** 4 Gy H (red), and **(F)** 8 Gy H (red). n ≥ 3, statistical analysis: paired t-test for whole curve or unpaired t-test for 96 h timepoint shown as (whole curve96 h) comparing treatment vs. 0 Gy control. p values > 0.05 (not significant, ns), < 0.05 (*), < 0.01 (**), and < 0.001 (***) were considered statistically significant.

long cytoplasmatic processes; nuclei appear to be increasingly anisomorphic. No morphological changes were seen following DoxA alone or in combination with any radiation treatment, compared with X or H irradiation alone. DoxB and DoxC, however, induced spindle-shaped cells with long cytoplasmic processes and anisonucleosis. Combined treatment with DoxB or C and both radiation qualities increased the amount of anisonucleosis and increased the frequency of cells, which lost their cytoplasmic processes and their bipolar spindled shape.

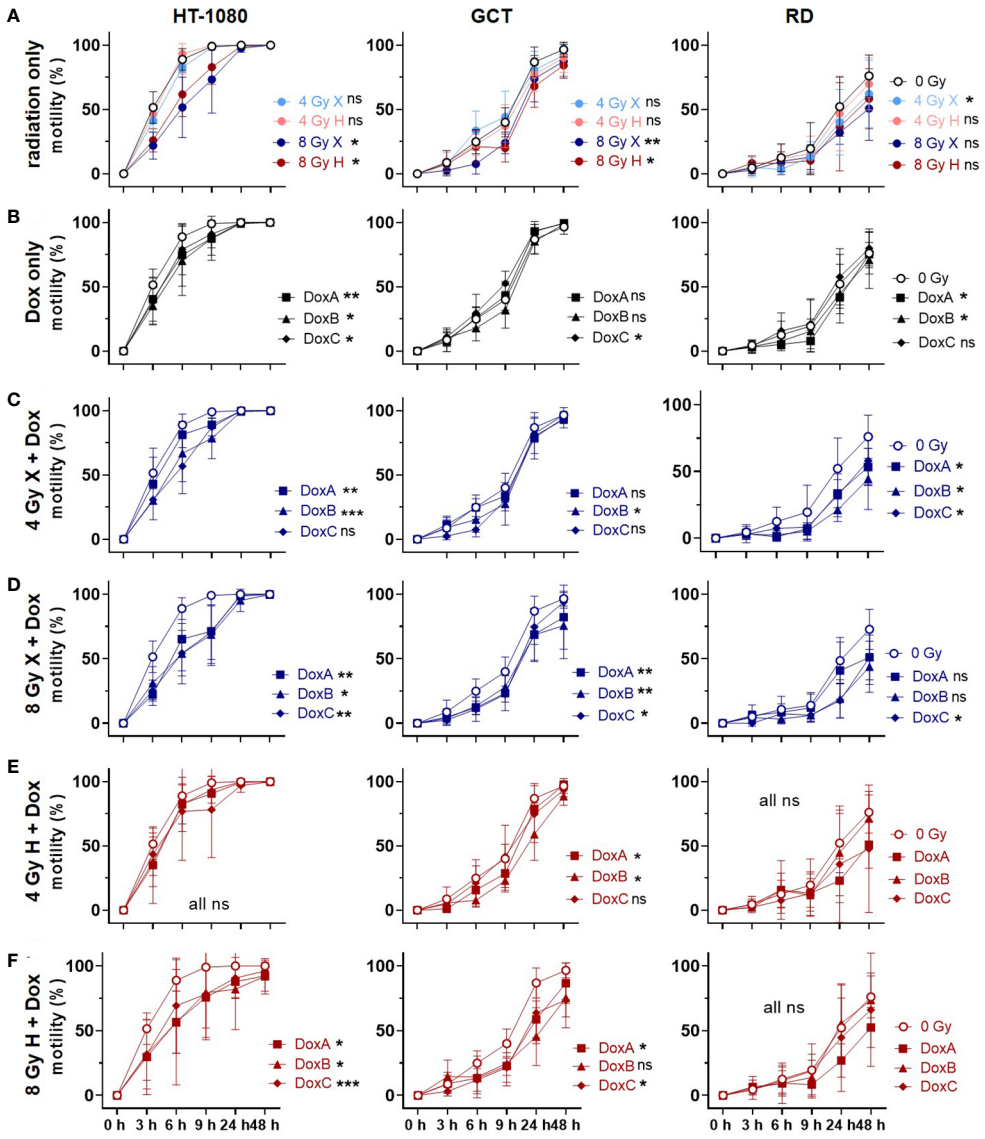
Unirradiated GCT cells show a largely homogeneous spindled morphology with long cytoplasmic processes creating intercellular connections. Following X and H irradiation, cells show cytoplasmic and nuclear enlargement; furthermore, multinucleated cells appear. Few GCT cells develop a dendritic shape with fibroblastic appearance. Monotreatment with DoxA or combined treatment with any radiation and DoxA did not alter the morphology. Cells under DoxB or DoxC treatment alone appear with extended cytoplasmatic processes in GCT cells. The morphological effects upon irradiation and chemotherapy alone were also seen following combined treatment with irradiation and DoxB or DoxC.

Untreated RD cells present as networking spindled cells with long cytoplasmic processes and broadly isomorphic nuclei. In co-localization, few single polygonal cells with larger, roundish nuclei are apparent. Upon X and H irradiation, cells and nuclei appear enlarged and increasingly anisomorphic, and multinucleated cells show up. The cytoplasm becomes granular, and some cells loose the spindled

morphology. Independent of treatment schedule, Dox treatment alone had no effect on the morphology of RD cells, like combined therapy with DoxA and radiation. However, exposure to combined X or H radiation and DoxB or C treatment led to the appearance of long thin processes, fibroblast-like and dendrite-like cell shapes, and increasing anisonucleosis. Overall, irradiation effects morphology of STS cells more pronounced than Dox treatment.

3.7 Cell motility: irradiation and Dox treatment reduced motility, but X-Dox was more effective than H-Dox

Finally, the migration assay was used to study cellular motilities by measuring the surface area that cells occupy over time after treatment with X or H irradiation and 10 nM Dox (Figure 6,



G

	0 Gy				4 Gy X				8 Gy X				4 Gy H				8 Gy H			
	no Dox	Dox A	Dox B	Do xC	no Dox	Dox A	Dox B	Dox C	no Dox	Dox A	Dox B	Dox C	no Dox	Dox A	Dox B	Dox C	no Dox	Dox A	Dox B	Dox C
HT-1080	17	13	12	13	14	14	10	10	7	7	10	8	15	12	13	15	9	10	10	11
GCT	5	6	3	6	9	4	2	2	2	2	3	3	4	5	1	5	5	1	0	3
RD	2	0.9	3	2	3	2	1	0.3	1	1	1	0	1	0	0	2	0.7	0	2	2

FIGURE 6
Migration assay comparing motility of 0 Gy control vs. treatment of HT-1080, GCT, and RD cells following **(A)** radiation only with 4 and 8 Gy X (light/dark blue) or 4 and 8 Gy H (light/dark red) radiation. **(B)** Dox treatment with10 nM DoxA (before), DoxB (before and after), or DoxC (after). Dox treatment schedule details in Figure 1D **(C–F)** Combined treatment with DoxA, DoxB, or DoxC and **(C)** 4 Gy X (blue), **(D)** 8 Gy X (blue), **(E)** 4 Gy H (red), and **(F)** 8 Gy H (red). **(G)** Maximum of migration speed for each cell line extracted from the exponential phase of the curve via linear regression. n ≥ 3, statistical analysis: paired t-test for whole curve comparison: treatment vs. 0 Gy control for HT-1080 cells until the scratch was closed (0–9 h) and for GCT and RD cells over the whole observation period of 0–48 h. p values > 0.05 (not significant, ns), < 0.05 (*), < 0.01 (**), and < 0.001 (***) were considered statistically significant.

Supplementary Figure 1). Relative to controls (0 Gy, 0 nM Dox), motilities for HT-1080 and GCT were significantly reduced following 8 Gy irradiation. However, RD cells lowered the motility only after 4 Gy X significantly (Figure 6A). Dox treatment lowered the motility in a cell line-dependent manner with HT-1080 being the most and GCT being the least affected (Figure 6B). Combined treatment of X irradiation and Dox reduced the motility in all cell lines where the 8 Gy dose was again more effective in HT-1080 and GCT, whereas RD was more affected after 4 Gy and Dox (Figures 6C, D). Interestingly, the combined treatment of Dox and H irradiation had less effects on cellular motilities. Only HT-1080 (8 Gy only) and GCT (both doses) cells showed significant effects (Figures 6E, F). The maximum speed of cell migration was calculated to form the exponential phase of the motility curves (HT-1080 cells 0–3 h; GCT cells: 3–6 h and RD cells 6–9 h). With the exemption of 4 Gy X in GCT and RD cells, the cell motility was reduced by radiation, all Dox schedules, and combined treatments relative to controls (0 Gy, 0 nM Dox, Figure 6G). The data were additionally normalized to the respective dose (4 or 8 Gy), radiation quality (X or H) (Supplementary Figure 8). Time- and dose-matching X and H irradiated samples were assessed to identify the potential influence of the radiation quality (Supplementary Figure 9). With the exemption of 4 Gy with DoxB in HT-1080 and 8 Gy with DoxC in RD, no significant influence of the radiation quality on the motility of the cells could be measured.

4 Discussion

In clinical practice, the established chemotherapy protocols of X-based radiochemotherapy (24) are adopted for H-based radiochemotherapy (8). Unfortunately, there is a lack of large clinical trials investigating the effects of combined H-based radiochemotherapy (25). In order to increase the body of preclinical data to optimize and improve established treatment protocols for combining radiotherapy, particularly with H and standard chemotherapy in STS, the effects of H irradiation compared with X irradiation and combined with the chemotherapeutic drug Dox in three different sequences in three STS models were evaluated.

In this study, the clonogenic cell survival, apoptosis induction, cell-cycle effects, proliferation, viability, morphological changes and cellular motility were investigated. It is shown that HT-1080 were the most radioresistant and RD the most radiosensitive cell lines (Figure 1C). GCT cells were most resistant to Dox treatment. For all cell lines, the longest Dox treatment (DoxB) showed the highest effectiveness (Figures 1E–G). The DoxC schedule reflects the treatment situation in the clinics where patients with low predicted overall survival benefit from adjuvant chemotherapy (12). DoxB and DoxC are superior to DoxA (Figures 1E-G), supporting the importance of the Dox treatment after radiation. Overall, the colony formation assay is the most relevant assay for the clinics because it investigates the long-term survival of STS cells. For all combined treatment scenarios, additive effects could be found. Overall, the combination of Dox and X seems to be more effective than Dox and H (Figures 1F, G).

The different STS cell types investigated (HT-1080 fibrosarcoma, GCT undifferentiated pleiomorphic sarcoma, and RD embryonal rhabdomyosarcoma cells) revealed a dose-dependent RT response with RD cells exhibiting the most radiosensitive phenotype followed by GCT cells and the quite radioresistant HT-1080 STS cells (Figure 1). Of note, no superior effects could be estimated for H versus X irradiation. Presented experiments were performed in the middle of the irradiation field (center of the SOBP), as this represented the predominant situation in the irradiation field of H therapy (26). However, the cell survival curves were not significantly different for all the investigated models (Figure 1A) and the determined RBEs were in the range of the clinical assumption of 1.1 (27). RBE values as low as 0.8 were found for survival level 1% (Figure 1B), which is indicative of a higher effectiveness of X radiation. Other groups have found increased RBE values representing a higher biological effectiveness of H irradiation in the distal fall-off of the Bragg peak (11). For other entities such as brain tumors, it has been discussed that the increased RBE at the end of the proton range can lead to increased side effects in healthy tissue (11). The clinical evidence for these effects remains weak (28). Therefore, future experiments should investigate the cellular response in this region of the treatment field. Striking was the linear curve progression of the RD cells. This indicates a decreased DNA damage repair capacity (29) of the cells and a high sensitivity to radiotherapy. In follow-up studies, the functional mutational status of DNA repair proteins should be clarified for this cell line. The α/β ratios of HT-1080 and GCT cells are also of interest (Figure 1B). Here, the ratios for H irradiation are higher in both cases. This could be an indication of reduced fractionation sensitivity of the cells (30).

Concerning the chemosensitivity of investigated STS cells, a pronounced chemotherapy sensitivity was estimated for each cell line. Significant differences between Dox mono treatment and Dox radiation were found for HT-1080 and RD cells. However, survival of GCT cells was not significantly altered in combined treatment relative to Dox monotherapy. When comparing the different Dox schedules, DoxB and DoxC were superior to DoxA. Dox and ifosfamide remain the most effective chemotherapy drugs available for STS tumors (31). However, management of STS is increasingly subtype-dependent and resistance for Dox is present. Resistance mediating molecular alterations such as the mutation of TP53 was discussed since p53-dependent apoptosis is the main mechanism of action of Dox (32).Unfortunately, the investigation of new molecular targets only showed an incremental progress and no superior effect relative to Dox (13). Nevertheless, patients with undifferentiated pleomorphic sarcoma (UPS, GCT cells) showed the highest overall response from treatment with monoclonal antibodies against PD-L1 (33). TP53 mutations are mostly associated with increased aggressiveness and radio resistance (34). The sarcoma cells studied here all showed to be positive for apoptotic cell death (Figure 2). However, apoptosis induction was significantly increased after radiation treatment compared with apoptosis after chemotherapy alone (Figures 2A, B). Mutations in the TP53 gene are known for the RD (homozygous mutation of TP53 (17)) and GCT (two heterozygous TP53 mutations (ATCC)) cell lines, whereas HT-1080 is proficient for TP53 (35). Especially in

GCT cells, no significantly increased apoptosis rates could be measured after treatment with Dox alone or in combination with H irradiation (Figures 2B–F). Nevertheless, it seems that the sequence of treatment has an impact on apoptosis rate and exclusive Dox treatment before irradiation is less effective than (before and) after radiation treatment (Figure 2B). Clinically, Dox chemotherapy is given as adjuvant or neoadjuvant intervention relative to radiotherapy. It is administered as a bolus injection within a few minutes or as a continuous intravenous infusion over several hours to days (36). The blood clearance of Dox varies widely inter-individually but extends over several days (36) so it can be assumed that Dox is present in sufficient amounts in tumor cells at the time of irradiation. All Dox experiments were performed in three different sequences with Dox treatment 3 h before irradiation (DoxA), 3 h before and refreshment within 1 h after irradiation (DoxB), or only within 1 h after irradiation (DoxC) (Figure 1D). Prolonged treatment with Dox in schedule DoxB or DoxC showed that major effects especially in combination with radiation additive effects could be determined (Figures 2B-F).

The mutation of RD and GCT cells for the TP53 gene is also reflected in the lack of p53-mediated G1/S cell-cycle arrest (37). Therefore, the cells temporarily arrest in the G2/M phase to repair DNA damage (38). In subsequent studies, the distribution and kinetics of DNA repair proteins might gain insight into the repair pathways used after X and H irradiation. First evaluations of decisive DNA repair protein levels however seemed to be unaltered in the STS cell lines investigated, at least under non-radiating conditions (data not shown). The increased repair of DNA damage via homologous recombination is intensively discussed in the context of H irradiation (38) and could be a starting point for the development of alternative drug therapy for STS.

Corresponding effects are demonstrated for cellular proliferations and viabilities after treatment (Figures 4, 5): All cell lines showed decreased proliferation activities and viabilities after irradiation (Figures 4A, 5A). For the Dox treatment alone, no effect could be determined in GCT cells for either endpoint, whereas effects for HT-1080 and RD cells were detected (Figures 4B, 5B). To investigate the additive effect of combined treatment in comparison with the mono-treatment with radiation (15), the data were normalized to matching controls (Supplementary Figures 5, 7). However, additive effects were determined for GCT cells on proliferation especially after combination with Dox and X, whereas the endpoint viability was not additively affected. These additive effects belong to the *in vitro* synergy, which differs from the therapeutic synergy (39).Taken together, these data suggest that GCT cells have to some extent a resistance to Dox and are most inactivated by irradiation. No particular sensitivity to a beam quality could be determined (Supplementary Figures 4, 6). In contrast, HT-1080 and RD cells are sensitive especially to prolonged Dox treatment and the combination with radiation, whether X or H, shows additive effects (Supplementary Figures 5, 7). Another important aspect is the different response of the cells in the cell viability and proliferation assay. While the proliferation of the cells is much more reduced following treatment (Figure 4A), the cell viability often recovers until 96 h (Figure 5A). Additionally, in the CFA with HT-1080 cells, single cells and colonies with less than 50 cells could be detected even after high-dose irradiation with 8 Gy, indicating that these cells are mitotically dead and stopped proliferating but are metabolically still active (40).

In all cell lines, radiation induced more changes in cell morphology compared with Dox treatment. However, no distinct differences in morphology between the radiation qualities (X or H) could be detected. Future work should include more time points, radiation doses, and additional treatment with relevant particles like carbon or oxygen ions to investigate the LET or RBE effect on morphology (41). Treatment-induced loss of bipolarity, prolonged cytoplasmic processes, and cell-shape alterations were seen for all cell lines indicating cell damage and cellular plasticity. For sarcoma, the transition from mesenchymal to (partial) epithelial (M(p)ET) cell type has been described and discussed as a potential biomarker for tumor treatment response (42). MET and the reversed-process epithelial to mesenchymal transition (EMT) have been discussed to contribute to doxorubicin resistance (43). Upregulation of EMT/MET genes has been reported, e.g., in rhabdomyosarcomas (44), which could be used for the development of new targeted drugs (45). The here found resistance for Dox of GCT cells corresponds with some gene analyses where genes, which are involved in chemoresistance (e.g., RAB22a and S100P), were upregulated in UPS. Furthermore, an upregulation of EMT-related genes and a downregulation of epithelial markers are common in UPS. The development of new drugs is ongoing. One example is eribulin, a novel microtubule inhibitor (45). Additionally, in context to rhabdomyosarcoma cases, upregulations of CDH1 (epithelial marker), SLUG (inducer of EMT), and MMP9 (matrix-modifying enzyme) are reported (44). In our study, no clear indications for MET or EMT could be seen based on cell cultures. To further analyze the potential cellular plasticity upon mono- or combined treatment, additional biomarker stainings for MET, e.g., N-cadherin, vimentin, and fibronectin, or EMT, e.g., E-cadherin, occludins, and claudins, are needed (46). To confirm the *in vitro* results, further investigations should be performed on tumor sections from *in vivo* or *in ovo* experiments. In our study, GCT and RD cells showed a pronounced resistance to Dox treatment of any schedule, which can in part be explained by the mutated TP53 gene. In primary STS cultures, a high mutation rate in apoptotic signaling genes (TP53, ATM, PIK3CB, PIK3R1, NTRK1, CSF2RB) was found and linked to Dox resistance (32). Future experiments should include molecular analysis regarding apoptosis and migration biomarkers to understand the additive effects mediated by anthracycline-based regimen. The involved genes and pathways could serve as new targets for personalized treatment approaches in sarcoma patients.

Finally, the migratory capacity of the three STS lines was investigated following the different radiation modality treatment with or without the different combined Dox schedules (Figure 6). For conventional X radiotherapy, an increased cell motility was shown, which holds the potential to promote invasion and metastasis (47). For the treatment of sarcoma, H radiotherapy is gaining importance (2). For example, for Ewing sarcoma cells (48), as well as for other cancer entities, e.g., for breast cancer cells (49), the enhanced motility following Dox treatment or X irradiation was already shown, but there is a lack of data for STS in general. Analysis of the motility in the three STS lines here revealed reduced migratory capacities following Dox and H treatment (Figures 6E, F).

In addition to apoptosis, the damage of the cellular membrane, which may influence the motility as well, is a further mechanism of action of Dox (22). Conclusively, the improved action of combined radiochemotherapy as investigated here not only improved the therapeutic response concerning cell survival but even reduced the migration/invasion potential especially following combined treatment with a prolonged sequence (DoxB or DoxC) (Figures 6C-F).

In summary, no clear advantage of H therapy over X therapy could be revealed in preclinical STS models. Experiments were performed in the center of the SOBP and not at the distal fall-off, where enhanced RBE values are described (10). RD rhabdomyosarcoma cells are quite radiosensitive followed by GCT undifferentiated pleiomorphic sarcoma cells. HT-1080 fibrosarcoma cells showed the highest radioresistance while being sensitive to Dox treatments due to wt TP53 (50). For the cell models used, prolonged Dox treatment was revealed as most effective. Combination of H radiations with Dox showed for most endpoints similar effects compared with X irradiation. Currently, the measured effects can be labeled as "cell line specific". To translate our findings to "STS subtype specific", more experiments with cells of the respective histology needs to be performed. Subtype-specific treatment approaches of STS increased constantly (13). A recent review summarized all published and publicly available STS cell lines and found only 45 histological subtypes represented in cell lines whereas 133 subtypes were not. For the here used histological subtypes, alternative cell models are available in sufficient numbers for fibrosarcoma and rhabdomyosarcoma, but not for undifferentiated pleiomorphic sarcoma/giant tumor cells (3). Conclusively, the presented findings strongly suggest that alternative drug therapies should be developed for combination therapy with H. The ultimate goal would be an individualized drug treatment tailored to the patient in combination with high-precision radiotherapy after (partial) surgical removal of the tumor.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

TB and CN conceptualized the study. Data acquisition was performed by TB, CB, and AK. CB calculated the proton beam fields for cell irradiation and AK analyzed the morphological changes following treatment. Analysis and interpretation of data were executed by TB, CN, and AK. Visualization was done by TB. DK provided protocols for proliferation, viability, and motility assay and provided temporary project supervision. The first draft of the manuscript was written by TB. CN and AK wrote parts of the manuscript. BT made proton beam time available. All authors revised the manuscript and approved the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1211984/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Exemplary images of migration assay with **(A)** HT1080 cells, **(B)** GCT cells, and **(C)** RD cells following 8 Gy X or H radiation and combined treatment with DoxB (before & after; details see) at different timepoints post scratch (0 h, 9 h, 48 h). 4 x magnification and scale bar = 553.3 µm.

SUPPLEMENTARY FIGURE 2
Flow cytometry comparing relative subG1 phase X vs. H of HT-1080, GCT, and RD cells following **(A)** radiation only with 4 and 8 Gy X (light/dark blue) or 4 and 8 Gy H (light/dark red) radiation. **(B-D)** combined radiation treatment with **(B)** DoxA (before), **(C)** DoxB (before & after), and **(D)** DoxC (after). Dox treatment schedule details in . $n \geq 3$, statistical analysis: paired t-test for whole curve or unpaired t-test for 96 h timepoint shown as (whole curve| 96 h) comparing matching X vs. H.

SUPPLEMENTARY FIGURE 3
Flow cytometry analysis of additive effects of combined treatment compared with monotreatment (radiation only) of relative subG1 phase (subG1) of HT-1080, GCT, and RD cells following combined treatment with 10 nM DoxA (before), DoxB (before & after), or DoxC (after) and **(A)** 4 Gy X (blue), **(B)** 8 Gy X (blue), **(C)** 4 Gy H (red), **(D)** 8 Gy H (red). Dox treatment schedule details in . $n \geq 3$, statistical analysis: paired t-test for whole curve or unpaired t-test for 96 h timepoint shown as (whole curve| 96 h) comparing monotreatment vs. combined treatment.

SUPPLEMENTARY FIGURE 4
CV assay comparing relative number of proliferating cells (proliferation; normalized to matched control) X vs. H of HT-1080, GCT, and RD cells following **(A)** radiation only with 4 and 8 Gy X (light/dark blue) or 4 and 8 Gy H (light/dark red) radiation. **(B-D)** combined radiation treatment with **(B)** DoxA (before), **(C)** DoxB (before & after), and **(D)** DoxC (after). Dox treatment schedule details in . n ≥ 3, statistical analysis: paired t-test for whole curve or unpaired t-test for 96 h timepoint shown as (whole curve| 96 h) comparing matching X vs. H.

SUPPLEMENTARY FIGURE 5
CV assay analysing the additive effects of combined treatment compared with monotreatment (radiation only) of relative number of proliferating cells (proliferation; normalized to matched control) of HT-1080, GCT, and RD cells following combined treatment with 10 nM DoxA (before), DoxB (before & after) or DoxC (after) and **(A)** 4 Gy X (blue), **(B)** 8 Gy X (blue), **(C)** 4 Gy H (red), and **(D)** 8 Gy H.(red). Dox treatment schedule details in . n ≥ 3, statistical analysis: paired t-test for whole curve or unpaired t-test for 96 h timepoint shown as (whole curve| 96 h) comparing monotreatment vs. combined treatment.

SUPPLEMENTARY FIGURE 6
WST assay comparing relative number of viable cells (viability; normalized to matched control) X vs. H of HT-1080, GCT, and RD cells following **(A)** radiation only with 4 and 8 Gy X (light/dark blue) or 4 and 8 Gy H (light/dark red) radiation. **(B-D)** combined treatment with **(B)** DoxA (before), **(C)** DoxB (before & after), and **(D)** DoxC (after). Dox treatment schedule details in . n ≥ 3, statistical analysis: paired t-test for whole curve or unpaired t-test for 96 h timepoint shown as (whole curve| 96 h) comparing matching X vs. H.

SUPPLEMENTARY FIGURE 7
WST assay analysing the additive effects of combined treatment compared with monotreatment (radiation only) of relative number of viable cells (viability; normalized to matched control) of HT-1080, GCT, and RD cells following combined treatment with 10 nM DoxA (before), DoxB (before & after), or DoxC (after) and **(A)** 4 Gy X (blue), **(B)** 8 Gy X (blue), **(C)** 4 Gy H (red), and **(D)** 8 Gy H (red). Dox treatment schedule details in . n ≥ 3, statistical analysis: paired t-test for whole curve or unpaired t-test for 96 h timepoint shown as (whole curve| 96 h) comparing monotreatment vs. combined treatment.

SUPPLEMENTARY FIGURE 8
Migration assay comparing relative number of motility X vs. H of HT-1080, GCT, and RD cells following **(A)** radiation only with 4 and 8 Gy X (light/dark blue) or 4 and 8 Gy H (light/dark red) radiation. **(B-D)** combined treatment with DoxA (before), **(C)** DoxB (before & after), and **(D)** DoxC (after) radiation. Dox treatment schedule details in . n ≥ 3, statistical analysis: paired t-test for whole curve comparing X vs. H (HT: 0-9h; GCT: 0-48h; RD: 0-48h).

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Current therapies and future prospective for locally aggressive mesenchymal tumors

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Locally aggressive mesenchymal tumors comprise a heterogeneous group of soft tissue and bone tumors with intermediate histology, incompletely understood biology, and highly variable natural history. Despite having a limited to absent ability to metastasize and excellent survival prognosis, locally aggressive mesenchymal tumors can be symptomatic, require prolonged and repeat treatments including surgery and chemotherapy, and can severely impact patients' quality of life. The management of locally aggressive tumors has evolved over the years with a focus on minimizing morbid treatments. Extensive oncologic surgeries and radiation are pillars of care for high grade sarcomas, however, play a more limited role in management of locally aggressive mesenchymal tumors, due to propensity for local recurrence despite resection, and the risk of transformation to a higher-grade entity following radiation. Patients should ideally be evaluated in specialized sarcoma centers that can coordinate complex multimodal decision-making, taking into consideration the individual patient's clinical presentation and history, as well as any available prognostic factors into customizing therapy. In this review, we aim to discuss the biology, clinical management, and future treatment frontiers for three representative locally aggressive mesenchymal tumors: desmoid-type fibromatosis (DF), tenosynovial giant cell tumor (TSGCT) and giant cell tumor of bone (GCTB). These entities challenge clinicians with their unpredictable behavior and responses to treatment, and still lack a well-defined standard of care despite recent progress with newly approved or promising experimental drugs.

KEYWORDS

desmoid fibromatosis, giant cell tumor of bone, tenosynovial giant cell tumor, locally aggressive mesenchymal tumors, malignant giant cell tumor of bone, metastatic giant cell tumor of bone, tyrosine kinase inhibitors, γ-secretase inhibitors

Introduction

Desmoid fibromatosis, giant cell tumor of bone and tenosynovial giant cell tumor are three distinct locally aggressive mesenchymal tumors with unpredictable behavior and absent to low tendency for malignancy (1). Historically, DF, GCTB and TSGCT have been managed following paradigms of treatment for high grade sarcomas with aggressive

surgeries and radiation treatment. However, important differences with respect to epidemiology, biology and prognosis between locally aggressive tumors and sarcomas have led to substantial changes in management over the last few years. Specifically, DF, GCTB and TSGCT affect predominantly young adults and, despite being locally aggressive and often highly symptomatic, have excellent prognosis (2–4). For all these reasons, and for the high rate of local recurrence, aggressive surgeries are no longer recommended. Similarly, radiation therapy is very rarely used nowadays for the risk of both malignant transformation and secondary cancer. The dismissal of aggressive treatments, the introduction of new drugs, the advancements in local treatment techniques, and better understanding of tumor biology have revolutionized the management of DF, GCTB TSGCT (5, 6). These diseases are now regarded more as chronic conditions in need of long-term symptoms and disease control without quality-of-life detriment. Patient associations and the expanding use of patient-reported outcome measures (PROMs) have largely contributed advancement in understanding the many physical, psychosocial, and practical challenges that patient encounter (7, 8).

Desmoid-type fibromatosis

Desmoid-type fibromatosis (DF), also known as aggressive fibromatosis, is a monoclonal fibroblastic neoplasm characterized by an infiltrative and locally aggressive growth pattern, high rates of post-surgical recurrence, and no metastatic potential (1).

Epidemiology. The incidence of DF is low with around 5 new cases per million people per year, with a peak between the 3rd or 4th decade of life and higher incidence in female patients (2).

Histopathology. Histologically, DF rarely cause diagnostic confusion, and are reliably comprised of bland hypochromatic spindled cells arranged in a densely fibrotic stroma (Figures 1A, B).

Etiopathogenesis. The etiopathogenesis of DF is not completely understood and likely multifactorial. Approximately 85-90% of DF cases are sporadic and harbor a mutation of the gene encoding the beta catenin protein, CTNBB1; whilst the remaining 5-10% of DF harbor an APC gene mutation and arise in the context of Familial Adenomatous Polyposis Syndrome (FAP) or attenuated FAP syndrome (9, 10). Key events in DF tumorigenesis are the genetic alterations of CTNNB1 or APC in sporadic or hereditary cases, respectively. Both mutations lead to constitutive activation of the Wnt/β-catenin pathway. In addition, Notch target genes have been shown to be overexpressed in DT and to engage in cross-talk with the Wnt/β-catenin signaling pathway, providing alternative potential therapeutic targets (11). Trigger events for tumorigenesis are thought to be a recent trauma, surgery, or pregnancy (12, 13).

Genetic testing. Molecular testing is encouraged as part of the diagnostic workup as virtually all DF harbor mutually exclusive mutations of either the CTNNB1 or APC genes (9, 14).

Clinical presentation. Clinically, DF can occur in any anatomic location. The vast majority of sporadic DF arise in the limbs, chest, and abdominal wall, while the intra-abdominal and head and neck location are less frequent. A previous surgery, trauma or recent pregnancy are common anamnestic findings and are frequently associated with *de novo* DF growth or progression of disease (1, 12, 13). FAP-associated DF harbor APC mutations can be multifocal and are frequently intra-abdominal. The diagnosis of APC mutated DF warrants FAP workup with colonoscopy and germline testing (9).

Natural history. The natural history of DF is unpredictable and can vary widely between patients; presenting symptoms depend on the growth rate and anatomic location of the tumor. Tumors can elicit severe symptoms when abutting nerves or vessels, or cause severe damage encompassing or invading intra-abdominal organs such as the bowel (15). In the last several years, the treatment approach has evolved considerably with emerging prospective evidence that long term stable disease and even spontaneous regression can occur in up to 20% of DF, even after an initial phase of growth (15–19).

Treatment

There is no standard of care for DF, which have been historically managed using similar paradigms to high grade sarcomas, with attempts at complete resection even at the cost of morbid surgeries, and various cytotoxic chemotherapies for unresectable tumors (15–19). The Desmoid Tumor Working Group (DTWG) is an international team of desmoid fibromatosis experts that in 2020 has issued evidence-based consensus guidelines with the aim of improving quality of care and patient's outcome worldwide (9).

Active surveillance. A "watch and wait" approach defined as "active surveillance" has been recommended by the DTWG for newly diagnoses patients, when the clinical presentation allows it, in view of the unpredictable behavior of DF and the high rate of spontaneous regression (9). Treatment initiation should be based on clear radiographic progression or emerging clinical symptoms (9). Patients managed with active surveillance should be monitored with imaging at 1 or 2 months from diagnosis then every 3 to 6 months. Progression in a single assessment in the absence of symptoms and when the tumor is in a non-critical location is not indication for treatment. Ideally, patients on active surveillance should be evaluated by an expert physician at a reference center for DF as the risk of progression may be high for large tumors (9).

When disease progression has been documented in at least two subsequent imaging assays, in the presence of worsening symptoms and for tumor arising in anatomical-critical locations, treatment should be considered. Systemic therapies should be favored over upfront surgical resection, which is now discouraged and reserved to few, selected cases due to preponderance of incomplete initial resections and frequent recurrences (9, 16–18, 20, 21).

Locoregional treatments

While surgery and radiation therapy (RT) are less and less employed, locoregional treatments such as cryoablation and high intensity focused ultrasound ablation have gained considerable interest over the past decade.

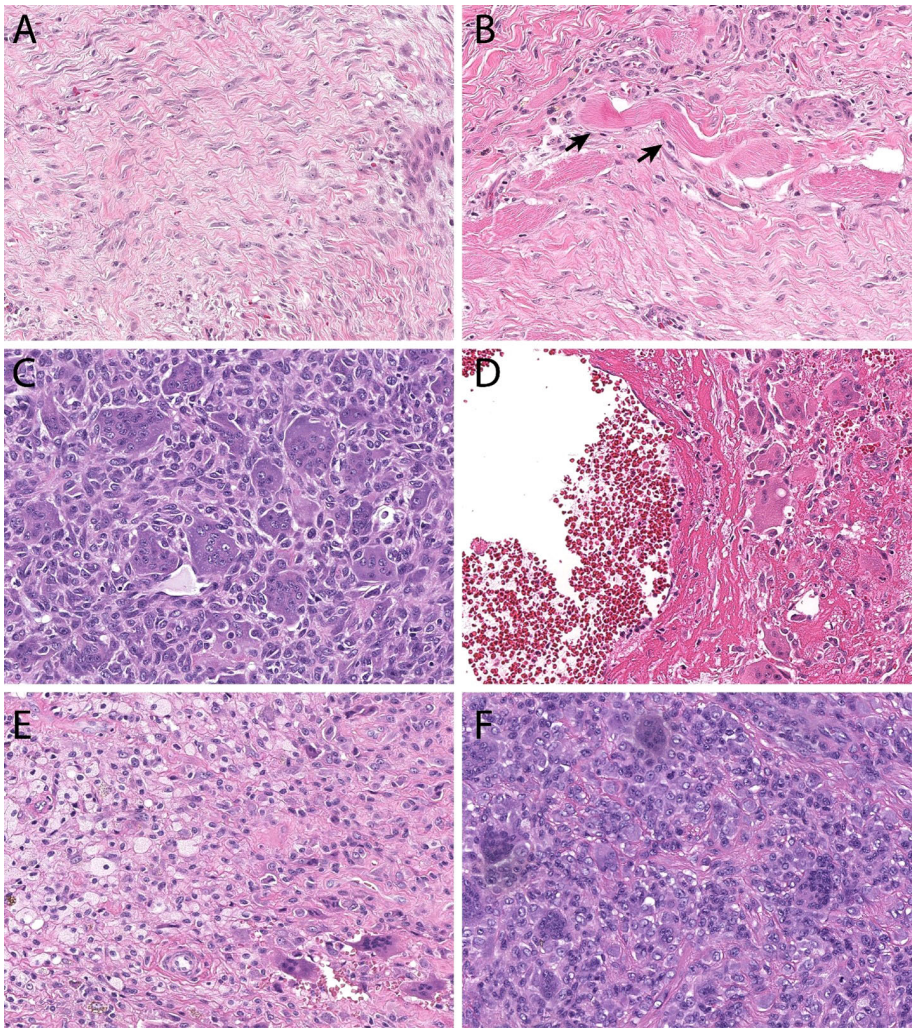

FIGURE 1
Histopathologic features. Desmoid-type fibromatosis **(A)** contains bland spindled cells arranged in a vague fascicular pattern. They often demonstrate skeletal muscle invasion (**B**, skeletal muscle fibers at black arrows), a finding that correlates with locoregional recurrence and incomplete excision. Giant cell tumor of bone **(C)** is comprised of monotonous mononuclear cells and an even distribution of osteoclastic giant cells. Both cell populations display similar nuclear features. In many instances, secondary aneurysmal bone cyst change **(D)** can be seen and can mask the underlying features. Tenosynovial giant cell tumor **(E)** is comprised of an admixture of foamy macrophages, osteoclastic giant cells, and inflammation. Monomorphic variants **(F)** can display increased cellularity, mimicking a sarcoma.

Surgery. Surgical resection of DF is no longer recommended as a first line treatment option, and it should be reserved for carefully selected patients (9). The high rate of local recurrence, difficulties on achieving negative margins along with the observed high rate of spontaneous tumor regressions are the reasons that led to the progressive decline of upfront surgery (2, 16, 17, 22). Resection can be considered for small DF of the abdominal wall whenever a complete tumor resection is deemed feasible without significant morbidity (21).

Radiation therapy. Radiation therapy is not routinely used in the management of DF and it should be avoided in the young population given the risk of secondary malignancy. Whilst retrospective series have failed to show statistically significant advantages in terms of local control when RT was used in combination with surgery versus surgery alone (23); moderate dose of RT can offer adequate local control (24). Overall, moderate dose RT can be considered in selected cases when systemic treatments are not effective and surgery is not feasible, especially for progressing tumors arising in critical locations as the head and neck region.

Cryoablation. This is a minimally invasive procedure in which a cryoprobe is percutaneously inserted into the tumor to deliver nitrogen or argon gas, inducing the formation of surrounding ice spheres and causing cell death through repeated cycles of freezing and passive thawing (25, 26). This modality of treatment has been increasingly used for DF of the extremity and trunk with several retrospective series showing encouraging data regarding safety and efficacy (25, 27, 28). Recent prospective evidence comes from the phase II clinical trial CRYODESMO-01 which reported that 86% of 50 previously treated patients had non-progressive disease and symptom improvement at 12 months post treatment (29). The vast majority of patients that undergo cryoablation experience

grade 1 or 2 toxicity including pain, redness, and swelling confined to the area of treatment, less frequently the formation of an hematoma or transient peripheral nerve damage is observed; serious adverse events are rare and include permanent nerve and neighboring structures or organs damage (29–31).

High intensity focused ultrasound (HIFU). A non-invasive local treatment that uses high frequency ultrasound waves to induce thermal coagulation of the target tissue. The procedure is performed under real time MR thermometry or ultrasound imaging to monitor the energy distribution and ensure sparing of surrounding tissues (32, 33). HIFU ablation is currently approved in the US for the treatment of uterine fibroids (34), prostate cancer (35), and for the treatment of painful bone metastasis (32, 36)with excellent results for symptoms control and functional results (37, 38). Retrospective evidence demonstrated successful employment of this modality of treatment for the management of desmoid fibromatosis (33, 39, 40). Iatrogenic complications of HIFU include grade 1 and 2 skin burns, and temporary nerve injury; less frequent although serious adverse events are ulceration and necrosis of non-target tissue caused by heat conduction and permanent nerve damage (33).

Medical therapy

Various systemic treatments are available for DF, and with the lack of a defined standard of care, the choice of which agent to use first is left to the treating clinician and institutional experience. Table 1 illustrates relevant clinical trials evaluating systemic treatment for DF (Table 1).

Antihormonal therapy. Antihormonal agents s such as tamoxifen or toremifene, alone or in combination with nonsteroidal anti-inflammatory drugs (NSAIDs), have been commonly used to treat DF (52, 53). Their employment was supported by the observed propensity of DF to arise during pregnancy and in the post-partum, and their frequent partial or complete regression after childbirth, supposedly as a consequence of estrogen levels returning to baseline (12, 13, 54–56). The biological rationale for using antihormonal agents comes from the proven estrogen receptor beta expression in 90% of DF (57) and their ability to prevent myofibroblasts differentiation (58). Antihormonal agents showed modest response rate across retrospective series (53, 59). About 30% of patients experience clinical benefit with tamoxifen with no clear correlation with radiological changes on MRI (60). It remains unclear whether the radiological findings and symptomatic improvement are treatment-induced or perhaps expression of the natural course of the disease and whether these drugs could have a role in the treatment of DF, especially when hormone or pregnancy related. Nowadays, antihormonal agents are no longer recommended for the lack of sufficient evidence supporting their use (DTWG).

Chemotherapy

Standard chemotherapy. Cytotoxic chemotherapy has been long used with evidence of efficacy deriving from several

TABLE 1 Main studies reporting on systemic treatment for DF.

Authors/ Study [ref]	Year reported	Diagnosis	Phase	Drug	Number of patients	Median age, years (range)	Endpoints	Outcome	p	Key points
Gega M. et al. (41)	2006	FAP associated DF	R	DOX/DTCI iv days 1-4 q28 followed by meloxicam	7	32.2 (28.1-37.1)	Toxicity PFS CR, PR, SD	No G4 74 mo 43%, 57, 0	NA	DOX/DTCI followed by meloxicam is safe and effective
de Camargo V. et al. (42)	2010	DF (32% with Gardner Sd, 44% intra-abdominal)	R	Anthracyclines, hormonal, MTX, imatinib	68	32.5	RR	50% doxorubicin 36% pegylated liposomal doxorubicin	NA	Anthacycline-containing regimens are associated with higher response rate compared to other chemotherapy combinations
Garbay D. et al. (43)	2012	DF (19.5% with Gardner Sd)	R	Mesna, adriamycin, ifosfamide, dacarbazine; Adriamycin, dacarbazine; doxorubicin; etoposide; MTX-vinblastine; vinorelbine; imatinib	62	30 (2–66)	RR CR, PR, SD PFS	54% anthracycline vs 12% 1.6, 19.4, 59.6% PFS 40.8 mo	0.0011	Anthacycline-containing regimens are associated with higher response rate compared to other chemotherapy combinations

(*Continued*)

TABLE 1 Continued

Authors/ Study [ref]	Year reported	Diagnosis	Phase	Drug	Number of patients	Median age, years (range)	Endpoints	Outcome	p	Key points
Constantinidou A. et al. (44)	2009	DF	R	pegylated liposomal doxorubicin (PLD)	11	29 (3- 53)	PR PFS	36% 14 mo	NA	PLD is effective and has acceptable toxicity profile
Ingley KM. et al. (45)	2019	DF	R	MTX/Vinorelbine	48	33 (13-73)	CR RECIST PR SD PD PFS tolerability	42% 39% 17% 2% 120 mo	NA	Highly effective, sustained response, minimal toxicity
Mir O. et al. (46) Long-term analysis	2020	DF	R	Oral vinorelbine single agent. Oral vinorebine and Hormonal treatment	100	35 (18- 67)	PR SD PFS 6, 12mo	29% 57, 88, 77%	NA	Oral vinorelbine is an effective, affordable, and well-tolerated regimen
Skapek SX. et al. (47)	2007	DF	II	Vinblastine and Methotrexate	26	11.4 (0.6 – 20.4 yo)	PR	31%	NA	Well tolerated. 5/26 pts had G4 neutropenia. First prospective trial in children
Chugh R. et al. (48)	2010	DF	II	Imatinib 300 mg BD orally	51	34 (12- 67)	CBR (clinical benefit rate as CR or PR within 16 weeks or SD lasting 16 weeks at least)	84%	0.24	RR to imatinib is low, PFS prolonged for some patients
Kasper B. et al. (49)	2017	DF	II	Imatinib 800 mg/d orally, treated planned for 2 years	38	44 (19- 80)	PAR_{6mo} PAR_{3mo} ORR	65% 88% 19%	NA	Imatinib induces sustained progression arrest
Gounder MM. et al. (20)	2018	DF	III	Sorafenib 400 mg/d orally	87	37 (28- 50)	PFS	2 y PFS 81% sorafenib vs 36% placebo	< 0.001	Sorafenib significantly prolonged PFS and induced durable responses
Gounder MM. et al. (50) DeFi trial	2022	DF	III	Nirogacestat 150 mg BID, placebo	142	34 (18- 76)	PFS Secondary: safety, ORR, PROs	71% risk reduction vs placebo (HR 0.29)	<0.001	Statistically and clinically significant improvement in PFS, ORR, health related QoL
Gounder MM. et al. (51) RINGSIDE trial Preliminary report	ongoing	DF	II/III	AL102- part A: 1.2 mg QD, 2 mg intermittent BIW (2 days on 5 days off), or 4 mg intermittent BIW	31 in part A, part B currently enrolling	40	PFS	ongoing	NA	ongoing

R, retrospective; DOX, doxorubicin; DTCI, dacarbazine; MTX, methotrexate; TKIs, tyrosine kinase inhibitors; Tem, Temozolomide; Horm, hormonal therapy; PFS, progression free survival; NA, not available; RR, response rate; ORR, overall response rate; CR, complete response; PR, partial response; SD, stable disease; RECIST, Response Evaluation Criteria in Solid Tumors; PAR_{6mo}, progression arrest rate at 6 months; PAR_{3mo}, progression arrest rate at 3 months; CBR, clinical benefit rate; QoL, quality of life.

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retrospective series and few prospective studies. Anthracycline-based regimes have significant activity in DF with response rate ranging from 37 to 54% (41–43). Patients are generally treated until satisfactory clinical response or when the maximum dose of anthracyclines is reached after 6 to 8 cycles (42). Potential toxicity from treatment include cardiomyopathy, especially when treatment is carried beyond the dose of 450mg/m2, and myelodysplastic syndrome (42). Pegylated liposomal doxorubicin has a reported response rate of 36% and better toxicity profile than its conventional form (44). Overall, anthracycline based chemotherapy regimens are effective and elicit rapid responses but have significant toxicity and should be reserved for selected patients only when a rapid response with prompt symptom control and tumor shrinkage are desired.

Low dose chemotherapy. Low dose chemotherapy with methotrexate (MTX) plus vinblastine (VBL) or vinorelbine (VNL) has been used especially in the young population (46, 47, 61, 62). Disease control is achieved after several months of treatment and response rate ranges between 35 to 40% (63). Late responses occur and contribute to the high long term-disease control with reported median PFS of 75 months and up to 136 months in patients that had responded to treatment (62). Low-dose MTX/VNL or VBL chemotherapy is effective and minimally toxic regimen but has significant impact on quality of life (QoL) for the lengthy duration of treatment. Single agent oral vinorelbine has a disease control rate of 86% with an excellent toxicity profile (46, 64). Low dose chemotherapy regimens are an effective, safe, and affordable choice that can offer long term symptoms and disease control, however responses are delayed compared to other agents; their use is especially common in the pediatric and young adults' population for the well understood toxicity profile.

Tyrosine Kinase Inhibitors. The clinical activity of tyrosine kinase inhibitors (TKIs) is well known, and several agents have been investigated in randomized controlled clinical trials. Imatinib, the first TKI evaluated for DF treatment, is effective on achieve disease control with 1 year progression free survival of 66% as confirmed by the results of two separate phase II trial (48, 65). Response to treatment is delayed compared to other agents with best responses seen at 19, 22 and 26 months with decreasing imatinib dosage of 600, 400 or 200 mg per day (48). The overall response rate (ORR) with imatinib is modest and even at the higher dose of 800 mg per day response rate observed is 19% (49). Sorafenib is a multitarget kinase inhibitor whose activity on DF has been extensively studied. The first evidence of efficacy came from the retrospective analysis of a cohort of 24 patients with clinical improvement in 16 (66%) and imaging confirmed partial responses in 5 cases (20%) (66). These observations prompted a more recent phase III placebo-controlled trial of sorafenib 400 mg per day against placebo. The two-year progression free survival was 81% in the treatment arm versus 36% in the placebo arm, while objective response rate for patient on sorafenib was 33% against 20% for placebo, confirming both the activity of sorafenib and quantifying the frequent spontaneous regression observed in DF (20). Pazopanib activity was retrospectively evaluated in a small cohort of 8 patients who received the drug at the starting dose of 800 mg with toxicity-led adjustments and final doses ranging from 200 to 800 mg/day. The overall observed PFS was 13.5 months with PR and SD according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 seen in 3/8 and 5/8 patients respectively (67). DESMOPAZ was a non-comparative, randomized, phase II trial that enrolled patients with DF to receive either pazopanib 800 mg per day or methotrexate and vinblastine chemotherapy. Partial response was seen in 37% of patients with a 6-months PFS of 83%; adverse events led to dose reduction for 73% of patients with fatigue, gastrointestinal toxicity and hypertension being most common (68). In summary, sorafenib and pazopanib are the most effective molecules with sorafenib being often favored in the clinical practice for the milder toxicity profile when compared to pazopanib.

Gamma secretase inhibitors. Recently, new drugs targeting the Wnt/beta-catenin and NOTCH pathways at different levels have been developed with encouraging evidence of efficacy both *in vitro* and *in vivo* (69–72). Reported results from the phase III placebo controlled DeFi trial showed promising activity of the gamma secretase inhibitor (GSI) nirogacestat in patients with progressive desmoid tumors (73). Nirogacestat treatment produced an overall response rate (ORR) of 41%, including 7% complete responses (CR), versus 8% in the placebo arm. Adverse events with nirogacestat were frequent but mostly low grade. Benefit was also measured *via* patient-reported outcomes, including improved pain, stiffness, and functional status (73). This agent is currently undergoing review after New Drug Application submission to the US FDA. Interim results of the phase II/III RINGSIDE trial of the GSI AL102 are also encouraging, showing a favorable toxicity profile and promising preliminary data of effective disease control (51). The beta-catenin inhibitor Tegavivint, which has proven *in vitro* antitumor activity, is currently being investigated in a phase I/II open label trial sponsored by the Children Oncology Group open to patients with recurrent or refractory desmoid tumors as well as other types of solid tumors (NCT04851119) (74, 75).

Future directions. Preclinical studies have implicated the epigenetic regulator EZH2, which is the catalytic subunit of the polycomb repressive complex 2, as a potentially druggable target. They observed *in vivo* inhibition of EHZ2 by tazemetostat with partial regression of autochthonous tumor models and *in vitro* activity of tazemetosat on Wnt pathway (76).

Areas of uncertainty. One of the main open questions remains how to properly select patients for which therapies. Many have postulated that the location of the driver mutation could influence the clinical course of the disease. Three recently reported studies suggested a trend toward worst outcome when the CTNNB1 mutation involves codon 45F, however the correlation failed to reach statistical significance (19, 77, 78). Similarly, mutational status does not correlate with response to treatment, but a correlation with worse general outcome has been observed for APC mutant and non-extremity DF (79).

Giant cell tumor of bone

Giant cell tumor of bone (GCTB) is a locally aggressive mesenchymal tumor with limited ability to metastasize, low rate of malignant transformation and high local recurrence rate (1).

Epidemiology. GCTB accounts for 3 to 5% of all bone tumors and generally occurs in young adults with peak incidence between 20 and 40 years of age (3, 80).

Histopathology. GCTB has unique histopathological features with a minor subset of stromal mononucleated osteoblast-like cells that are thought to be responsible for the growth and survival of a second population of multinucleated, osteoclast-like giant cells. The neoplastic stromal osteoblastic cells produce chemotactic factors including nuclear factor kappa B ligand (RANKL). Increased levels of RANKL promote pathologic recruitment of monocytes to the tumor site and induce their differentiation into osteoclasts-like giant cells, ultimately responsible for the osteolysis seen in GCTB (Figures 1C, D) (81–83).

Tumor classification. There are two subtypes of GCTB: a more common conventional type, and a primary malignant GCTB, a rare entity accounting to less than 2% of new diagnoses (84, 85). Moreover, 2 to 3% of conventional GCTB can undergo sarcomatous transformation into a malignant tumor, in most cases several years after radiotherapy or curettage (86–88). Conventional GCTB can rarely metastasize, this occurs in less than 10% of patients with the lung being the most common site of secondary disease. Pulmonary involvement tends to remains asymptomatic, and it is not necessarily linked to malignant transformation (89).

Clinical presentation. GCTB predominantly arises from long bones such as femur and tibia, especially around the knee, but it can also affect the pelvis, smaller bones of feet and hands, and other less typical locations (90). Clinically, GCTB can cause pain, swelling, deformity, and loss of function depending on the site of disease; if left untreated, GTCB can lead to bone resorption, fracture, and neurological symptoms (91, 92).

Local treatments. The mainstay of treatment for GCTB is aggressive curettage or surgical en-bloc resection of the affected bone, while medical treatment is reserved for recurring or unresectable tumors and in lieu of morbid surgical procedures (92). Intralesional curettage with allograft or bone cement reconstruction is a widely accepted procedure that allows local control without sacrificing function (91–93). Local recurrence rate after curettage is high, ranging between 25 and 50% with conflicting reported data regarding the impact of different bone reconstruction techniques and filling materials (91, 92, 94–101). Peri-surgical interventions have been explored with the intent to lower the rate of local recurrence with no evidence of benefit so far (102, 103). Adjuvant radiotherapy may decrease the chances of post-surgical recurrence, but it is known to induce secondary malignant transformation, making it not a commonly pursued treatment (86, 87, 104–106).

Medical treatment

RANK ligand inhibitors. Denosumab is a fully human IgG2 monoclonal antibody that binds RANKL, preventing it to interact with his receptor, RANK, on the surface of osteoclasts and their precursors. Reduced RANL-RANK binding inhibits osteoclasts formation, function, and survival, ultimately controlling osteolysis and inducing ossification and fibrosis (107–111). The first proof of concept study of denosumab on GCTB was an open-label phase II study that enrolled 37 patients with recurrent or unresectable tumors to receive subcutaneous denosumab 120 mg every 4 weeks with additional doses on day 8 and 15. The primary endpoint was the proportion of patients with a tumor response at 25 weeks defined as histopathological confirmed elimination of 90% of giant cells; or, where giant cells represented less than 5% of tumor cells at baseline, complete elimination. Non radiological progression was used to estimate efficacy when histopathologic data were not available. Of 35 assessable patients, 30 had either histological or radiological response (112). Later analysis of tumor specimens confirmed that denosumab significantly reduces RANK-positive tumor giant cells, as well as the relative proportion of proliferative, densely cellular tumor stroma, and promotes the formation of differentiated bone tissue (108). A larger phase II study enrolled 282 patients distributed in three cohorts to receive denosumab at the established dose of 120mg subcutaneously every 4 weeks with extra doses on day 8 and 15 of the first cycle. Patients in cohort 1 (n = 169) had inoperable disease and received denosumab as the only treatment. Patients in cohort 2 (n= 100) received neoadjuvant denosumab for salvageable GCTB, these patients had GCTB that were deemed resectable with technically feasible, but potentially morbid surgical resections. Cohort 3 included patients who were transitioned from the previous phase II study. Results from interim analysis after a median follow up of 13 months showed that 96% of patients from cohort 1 had non-progressive disease; seventy-four percent of patients from cohort 2 had not undergone surgery and, among the 26 patients who did, 16 had a less morbid procedure than initially planned. Toxicity included joint pain in 20% of patients followed by headache, nausea, back pain, and fatigue; osteonecrosis of the jaw (ONJ) was seen in 1% of patients (113). Based on demonstrated efficacy, denosumab was approved by the FDA in June 2013 for its use in adults and skeletally mature adolescents with giant cell tumor of bone deemed unresectable or requiring morbid surgery or in metastatic disease. Long term follow up data of the same trial was analyzed for safety and efficacy and published in 2019, after the enrollment was expanded to include a total of 532 patients. The median follow up was 58 months for the overall population, 65.8 months for cohort 1 and 53.4 months for cohort 2; at the time of the analysis 11% of patients in cohort 1 had progressed and 92% of patients in cohort 2 had not undergone surgery in the first 6 months of treatment. Common G3 or G4 toxicity were hypophosphatemia (5%), osteonecrosis of the jaw (ONJ) (3%), atypical femoral fracture (1%); 1% of patients presented with hypercalcemia occurring 30 days after discontinuing treatment (114).

The role of denosumab in the neoadjuvant setting has been also evaluated, with conflicting results so far (102, 103, 115). A later analysis of the expanded cohort 2 of the above-mentioned trial evaluated the potential impact of pre-operative denosumab on downstaging surgery. A total of 222 patients candidate to extensive surgeries (hemipelvectomy, amputation, joint replacement/fixation) were treated with denosumab for a median duration of 15.3 months; at the date of cutoff for data analysis, 48% of patients had not yet undergone surgery, while 38% of them had been able to undergo less morbid surgeries than originally planned. In this study, 17 of the 116

surgical patients experienced disease recurrence after a median time of 13.6 months. Notably, the median follow up post-surgery was 13 months, hence the results may underestimate the actual rate of local recurrence (116). Further evidence supports the use of denosumab for patients with unresectable disease as well as in the neoadjuvant setting, as it may facilitate surgery and allow avoidance of mutilating resections (117–119).

Data suggests that the combination of neoadjuvant denosumab and curettage is associated with a high risk of disease recurrence. Errani et al. reported local recurrence rate as high as 60% (15/25) for patients who underwent curettage after receiving denosumab versus 16% recurrence rate (36/222) for patients treated with upfront curettage (120). This was confirmed by several groups and by a recent metanalysis that showed that tumors treated with denosumab plus curettage have a relatively higher risk of recurrence compared with tumors managed with curettage alone (P = 0.07) (102). It has been postulated that denosumab-induced tumor changes may be responsible of the higher recurrence rate; for example, the development of a peripheral calcified rim that can preclude radical curettage as well as the persistence of latent tumor cells in the new formed bone may represent the cause of recurrence and may require more aggressive curettage (108, 121, 122). This is supported by the knowledge that denosumab targets the osteoclastic cells and lacks antitumor effect against neoplastic stromal cells that can restart proliferating when the RANKL Ab disappears from the microenvironment, as proven by *in vitro* evidence (123).

The short-term efficacy of denosumab and toxicity profile at the standard dosing in patients with unresectable GCTB are well described. However, patients with unresectable GCTB are, by definition, candidates for prolonged treatment that can lead to drug related complications. On the other hand, discontinuation may be followed by disease relapse (124). In a cohort of 54 patients with unoperable or metastatic GCTB, ONJ was observed in 9% of patients, skin rash and hypophosphatemia in 11 and 4% respectively. Ten patients discontinued denosumab and were followed up for a median time of 15 months; 4 of them had disease progression after 7 to 15 months from treatment discontinuation, while 6 had no signs of active disease months to a few years after treatment cessation (124). Rebound hypercalcemia with acute kidney injury 5.5 to 7 months post denosumab was described in three young patients (14, 15 and 40 years) who had been treated for 1.3 to 4 years and stopped treatment for toxicity (125). This prompts new questions regarding the optimal length of treatment with evidence that some patients may be able to discontinue denosumab and enjoy sustained response, while other may need longer treatment (124, 126).

Increasing interval of denosumab dosing and establishing the optimal length of treatment may help find a balance between satisfactory disease control and avoidance of serious adverse events. Effects of increased dosing interval has been evaluated in a retrospective cohort of 37 patients. Dosing interval was increased for 38% of patients with most common final interval of 12 weeks, this resulted in similar tumor control compared to standard dosing and lower absolute number of bone toxicity events (127). The rationale supporting longer interval is that the half-life of denosumab is 32 days and the inhibitory effects on osteolysis lasts 12 weeks (128, 129). The REDUCE trial, whose results are awaited, was designed to investigate risks and benefits of maintenance treatment with reduced intensity denosumab after 12-15 months of conventional dose treatment in patients needing long term therapy (130). Overall, denosumab provides long term disease control for patients with unoperable GCTB and its use is now well established. Conversely, the decision of initiating medical treatment for patients with operable GCTB should be pondered and the selected surgical modality defined prior to the start of systemic treatment. In fact, although denosumab may improve the outcome for patients undergoing en-bloc resections, it can increase the risk of local recurrence in case of intralesional curettage. Therefore, surgical and medical treatment planning for GCTB should be coordinated by a sarcoma multidisciplinary team.

Tyrosine Kinase Inhibitors. Lenvatinib is a multitargeted tyrosine kinase inhibitor whose effect on GCTB patient derived 2D and 3D primary culture was tested in a recently reported study. Five patients derived primary GCTB series were exposed to denosumab, lenvatinib and a combination of denosumab and lenvatinib. Interestingly, lenvatinib exhibited higher activity both in 2D and 3D compared to denosumab (131). The involvement of VEGFR has been described in supporting RANKL-induced osteoclastogenesis in GCTB and the above results confirm the promising role of antiangiogenic drugs in its management (131, 132). Table 2 illustrates relevant clinic trial assessing systemic treatment for GCT (Table 2).

Malignant giant cell tumor of bone

GCTB can rarely undergo malignant transformation and acquire histopathological characteristics that are similar to a high-grade sarcoma such as undifferentiated sarcoma or osteosarcoma (145). Malignant transformation is reported in 1 to 4% of patients; malignant GCTB are classified as primary malignant (PMGCTB), secondary malignant GCTB (SMGCTB) or GCTB with sarcomatous transformation not secondary to treatment (84, 146). In primary malignant GCTB, distinct areas of benign GCTB are juxtaposed with high-grade sarcoma ones, making it a challenging and often missed diagnosis (85, 147). The radiologic features of PMGCTB are also similar to those of conventional GCTB presenting as osteolytic lesions with well-circumscribed margins (84, 85, 148). In secondary malignant GCTB (SMGCTB), malignancy is diagnosed at the site of conventional GCTB previously treated with radiation or surgery (147, 148). Malignant transformation in GCTB after or during treatment with denosumab has also been reported; however it remains unclear whether denosumab can favor malignant transformation through immunosuppression or if at least some progressive SMGCTB were malignant tumors initially misdiagnosed (111, 113, 114, 116, 146, 149–152). Sarcomatous transformation of conventional, treatment naïve GCTB has been sporadically observed (146). Latency between the primary diagnosis of conventional GCTB and malignant GCTB can vary between 3 to over 20 years according to historical data (146, 147, 153).

TABLE 2 Main studies reporting on systemic treatment for GCTB.

Authors/Study [ref]	Year reported	Diagnosis	Phase	Drug	Number of patients	Median age, years (range)	Endpoints	Outcome	p	Key points
Thomas et al. (112) First study of denosumab for GCT	2010	GCTB	II	Denosumab 120 mg sc q4w with extra dose day 8 and 15	37	34 (22- 46)	RR at 25 weeks (elimination of at least 90% of giant cells or no radiological progression of the target lesion)	86% 30/35 responders 20/20 histologically 10/15 radiologically	NA	Denosumab elicits histological and radiological response
Chawla et al. (113) Interim analysis	2013	GCTB	II	Denosumab 120 mg sc q4w with extra dose day 8 and 15	282 in 3 cohorts	33.5	safety profile TTP -cohort1 Time to surgery -cohort2	96% at 13 months 74% no surgery at 9.2 months	NA	Denosumab was associated with tumour responses and reduced the need for morbid surgery
Martin-Broto et al. (6) Interim results of the previous study	2014	GCTB	II	Denosumab 120 mg sc q4w with extra dose day 8 and 15	281	33.5	Proportion of patients with clinically relevant decrease in worst pain Time to decrease in pain Time to increase in pain	Cohort 1,2, and 3: 29% and 35% 77% and 79% 30 and 15 days 6.9 and 30%23.2 months- N/A	NA	Rapid and clinically relevant pain relief
Rutkowski et al. (116) Analysis of cohort 2 from phase 2 trial from Chawla 2013	2015	GCTB	II	Denosumab 120 mg sc q4w with extra dose day 8 and 15	222	34 (25-44)	Cohort 2 patients for surgical downstaging rate	48% had no yet undergone surgery at cutoff time; 38% had less morbid surgeries	NA	Beneficial surgical downstaging, including either no surgery or a less morbid surgical procedure
Chawla S. et al. (114) Long term follow up	2019	GCTB	II	Denosumab 120 mg sc q4w with extra dose day 8 and 15	532	33 (25- 45)	Primary: Safety Secondary: PFS for cohort 1; percentage of patients not undergoing surgery for cohort 2	PFS not reached at the preliminary analysis	NA	Denosumab is safe and shows long term disease control
Bukata et al. (133) Subanalysis of phase II from Chawla 2013	2019	GCTB of the spine including sacrum	II	Denosumab 120 mg sc q4w with extra dose day 8 and 15	132	32 (13–83)	Safety Efficacy with estimate of PFS For patients in cohort 1	- 3% and 7.4% at 1 and 3, 5 years	NA	Safe and potentially useful for GCTB of spine and sacrum
ClinicalTrials.gov Identifier: NCT03620149	2021	GCTB	II	Denosumab maintenance 120mg SC 12-weekly (after 12-15 months at conventional dosing)	NA	NA	PFS ONJ	Not reported	Not reported	Not reported
Jiang et al. (127)	2022	GCTB	R	Denosumab 120 mg sc at various increased interval, most commonly 12 weeks	37	37 (22-73)	Difference in efficacy and bone toxicity or standard dose vs increased interval	No difference in efficacy, toxicity, mPFS.	NA p= 0.22 p= 0.97	Tumor control is similar, bone toxicity is better with enlarged intervals

(Continued)

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TABLE 2 Continued

Authors/Study [ref]	Year reported	Diagnosis	Phase	Drug	Number of patients	Median age, years (range)	Endpoints	Outcome	p	Key points
							Median PFS 5-year PFS	5 y PFS was longer for less frequent dosing	p= 0.036	

R, retrospective; PFS, progression free survival; mPFS, median progression free survival; RR, response rate; ORR, overall response rate; CR, complete response; PR, partial response; SD, stable disease; RECIST, Response Evaluation Criteria in Solid Tumors; CBR, clinical benefit rate; QoL, quality of life; NA, not available.

Overall, malignant GCTB is associated with poor outcome, with post-radiation SMGCTB showing an especially aggressive behavior (147, 153). Malignancy should be suspected in case of pulmonary involvement, poor response to denosumab, aggressive clinical behavior and disease that recurs after a latency period of more than 4 years (84, 148). Surgical resection is the mainstay of treatment for malignant GCBT (154). Although adjuvant chemotherapy has failed to improve the overall survival for patients with malignant GCBT, it seems associated with longer pulmonary metastasis free survival (148).

Metastatic GCTB

Metastatic disease is rare and typically involves the lungs. The pathophysiology of pulmonary metastasis of GCT has not been determined, and various factors from tumor vascular invasion to iatrogenic embolization have been suggested as the cause pulmonary spread (155). Pulmonary metastases have matching histological features to the primary tumor, are generally indolent and not necessarily linked to malignant transformation, however the incidence of lung metastasis is high for malignant GCTB (148, 156). The observed interval between primary diagnosis and development of pulmonary metastasis is significantly shorter for malignant GCTB compared to the conventional type (9 vs 21 months) according to a large retrospective case series reported by Liu et al. (148). The incidence of lung metastasis seem to be influenced by the presence of malignancy, time to recurrence, time for primary diagnosis and tumor size (157). The clinical course of pulmonary metastatic disease is unpredictable. Lung metastasis may be managed with surveillance at first, however about 50% of patients will eventually experience disease progression and need treatment with metastasectomy or denosumab (158). Overall, the prognosis of patients with metastatic disease is favorable but many questions remain open including surveillance recommendations, risk stratification and best management of disease.

Tenosynovial Giant Cell Tumor

Tenosynovial Giant Cell Tumor (TSGCT) is a rare, locally aggressive neoplasm that arises from the synovium of joints, bursae, and tendon sheaths (4, 6).

Epidemiology. The incidence of TSGCT is estimated to be of 1.8 cases per million per year in the USA, with a peak between 30 and 50 years of age and female prevalence (3, 159–161).

Histopathology. TSGCT is characterized by elevated expression of the colony-stimulating factor (CSF1) gene (97). Several mechanisms leading to CSF1 hyperexpression have been described such as translocations or deletions, the vast majority of them resulting on exon 9 deletion, which negatively regulates CSF1 expression (134, 162, 163). This causes overexpression of CSF1, responsible for the recruitment and growth of CSF1R expressing monocytes and drives the development of a tumor formed by a large number of nonneoplastic macrophages expressing CSF1R and a

minority of neoplastic cells, which do not express CSF1R (134, 162–164) (Figures 1E, F).

Tumor classification. TSGCTs are classified in two distinct subtypes based on growth pattern and presentation: localized or nodular type (N-TSGCT) and infiltrative diffuse type (D-TSGCT). Although D-TSGCT displays an infiltrative border, both subtypes are strikingly similar microscopically, being comprised of an admixture of cell types without significant cytologic atypia (Figures 1E, F). N-TCGT, the most common subtype, arises from digits in 80% of cases with less frequent locations being the wrist, ankle, foot, knee and, even more rarely, large joints. D-TCGT is rare and affects the knee in 75% of observed cases, followed by the hip, elbow, shoulder and ankle (81). An extra-articular form D-TSGCT is possible, with tumor growth within the peri-articular soft tissue and no evidence of articular involvement (165). Malignant TSGCT is exceedingly rare and affects people between 50 and 60 years of age; is characterized by areas of sarcomatous differentiation and tends to metastasize to the lymph nodes and lungs rather than locally recur (161, 166–170).

Clinic and natural history. TSGCT has an excellent prognosis, and, with the exception of the rare malignant form, it is not considered a life-threatening disease (171). Clinically, N-TSGCT tends to have an indolent course, while D-TSGCT is more aggressive and can have variable behavior from paucisymptomatic to severely symptomatic disease with joint pain, swelling, locking, instability, numbness, diminished range of motion and decreased quality of life. Not all patients experience symptoms, and for this reason management should be individualized and the clinical presentation must be considered when deciding between active surveillance versus systemic or surgical treatment (172, 173).

Local treatments

Surgery. In case of symptomatic disease, surgery is the primary treatment for both subtypes. However, there is growing consensus on wanting to avoid morbid resections and consider systemic treatment instead (6). Most N-TSGCT can be cured with marginal resection, whilst D-TSGCT require extensive synovectomy and, despite this, have a chance of local recurrence reported between 30 and 50% with even higher rate for repeat resections (171).

Radiation therapy. Peri-operative interventions with systemic treatment or radiotherapy are not standard of care although considered by some authors for borderline operable cases (174).

Medical treatment

CSF1R inhibitors. Improved insight into tumor biology has revolutionized systemic treatment and several molecules targeting CSF1/CSF1R have successfully been employed. Pexidartinib is an orally available CSF1R inhibitor approved in the USA for the treatment of adults with inoperable and severely debilitating tumors (164, 175). Evidence that brought to the approval of pexidartinib comes from a phase III study against placebo showing an overall response rate of 39% in the treatment arm at week 25 versus 0% for placebo, as well as improvement in patient-reported outcomes, including scores for pain, stiffness, and function (135, 175). To assess the long-term effects of pexidartinib, a pooled analysis of studies ENLIVEN and the TSGCT cohort of the PLX 108-01 study was performed by Gelderbom et al. (136). The study population consisted of a cohort of 120 patients treated with pexidartinib; ORR was 60% according to RECIST and 65% according to Tumor Volume Score (TVS) measurement, 77% of responses occurred within 6 months from treatment start, and the median duration of treatment was 19 months. Regarding toxicity, 68% of patients experienced adverse events (AEs) requiring dose reductions or treatment discontinuation; 92% had aminotransferase elevation between 1 and 3 x ULN in 66% of cases, while 4 patients had mixed cholestatic hepatotoxicity which resolved within 1 to 7 months from drug interruption (136).

Imatinib. Imatinib mesylate blocks the CSF1R and is active against TSGCT. Evidence of efficacy comes from a large multicenter retrospective study that included 58 patients treated with imatinib for advanced symptomatic, recurrent, or metastatic (2 patients) TSGCT. The response rate (RR) among all patient was 31%, PFS was 18 months, patient reported clinical benefit was favorable as well as the toxicity profile (137, 143).

Nilotinib. Nilotinib, a tyrosine kinase inhibitor active against CSF1, has shown short-term disease control with 90% of PFS at 12 weeks, and mixed long-term disease control with PFS of 52% at 5 years (139, 140). Further data from a recently completed phase II study of Nilotinib in patients with relapsed or metastatic TSGCT are awaited and will help clarify the role of Nilotinib for TSGCT treatment (NCT01207492).

Ongoing clinical trials. New agents are also currently being studied in ongoing clinical trials. ***CSF1R inhibitors.*** ViImseltinib is an oral CSF1 inhibitor currently investigated on the ongoing phase III MOTION trial (NCT05059262). Recently reported results from phase I and phase II trials show that all enrolled patients benefited from treatment in terms of symptoms or disease control with manageable toxicity profile (144, 176).

Monoclonal antibodies against CSF1R. Monoclonal antibodies against CSF1R cabiralizumab and emactuzumab have been studied on patients with D-TSGCT with preliminary evidence of efficacy from phase I/II trials (138, 141, 142). Results from a recently completed phase III trial of emactuzumab are awaited (NCT05417789).

Class effect toxicities of CSF1/CSF1R inhibitor including hypertension, oedema, and liver toxicity can rarely be serious. In the attempt to avoid systemic toxicity and successfully treat this localized disease, a trial of intra-articular administration of the CSF1 receptor antibody AMB-05X is ongoing (NCT05349643). (Table 3) illustrates relevant clinic trial assessing systemic treatment for TSGCT.

Discussion

Despite progress made in systemic and local treatments and improved understanding of disease biology, patients with locally aggressive mesenchymal tumors still may experience unsatisfactory

TABLE 3 Main studies reporting on systemic treatment for TSGCT.

Authors/ Study [ref]	Year reported	Diagnosis	Phase	Drug	Number of patients	Median age, years (range)	Endpoints	Outcome	p	Key points
Tap W. et al. (134)	2015	TSGCT	I/II	Pexidartinib po 1000mg/d	20	46 (22- 80)	Clinical benefit CR/PR/SD	95% 0/12/7	NA	Prolonged regression of tumor
Tap W. et al. (135) ENLIVEN	2019	TSGCT	III	Pexidartinib po 1000mg/d vs placebo po	120	44 (22- 75)	RR at 25 weeks CR/PR	39% vs 0 15%/25%	0.0001	Robust tumor response with improved symptoms; mixed or cholestatic hepatotoxicity is an identified risk.
Gelderblom et al. (136) Long term effects of pexidartinib	2021	TSGCT	Pexidartinib pooled analysis	Pexidartinib po 800-1000mg/d	130	45 (20- 80)	Best overall response by RECIST (CR, PR) DOR by RECIST	78% Not reached	NA	Overall LT benefit of continued treatment with pexidartinib
Cassier PA. et al. (137)	2012	TSGCT	R	Imatinib 400 mg/ day orally	27	41 (21- 77)	RR SD CR/PR	19% 74% 1/4	NA	Potential effect of imatinib on targeting CSF1
Cassier PA. et al. (138) Emactuzumab Phase I long-term	2015	TSGCT	I	Emactuzumab IV 900-2000mg/2weeks	28	42 (18- 82)	Safety RR CR	86% 7%	NA	Promising activity, 5 serious adverse events
Gelderblom H et al. (139)	2018	TSGCT	II	Nilotinib 800mg/ day orally	56	36 (18- 74)	PFS 12 weeks	94%	NA	Manageable toxicity and good disease control at 12 weeks
Spierenburg G et al. (140) Long-term Nilotinib Ph II	2022	TSGCT	II	Nilotinib 800mg/ day orally	48	37 (23- 51)	LT-PFS Duration of response mTTP clinical worsening LT- toxicity	48%	NA	Mixed effect of nilotinib with half of patient needing nire treatment at 8.5 years follow up
Sankhara KK. et al. (141)	2017	D-TSGCT	I/II	Cabiralizumab 1, 2, 4mg/kg	22	not reported	Safety efficacy	not reported	Not reported	Not reported
Cassier PA. et al. (142)	2020	D-TSGCT	I	Emactuzumab IV 900-2000mg/2weeks	63	38 (18–82)	Safety RR CR/PR	71% 3%, 68%	NA	Manageable toxicity, durable response
Verspoor FGM et al. (143)	2019	TSGCT	R	Imatinib 400 mg/ day orally	58	45 (36–56)	RR CR/SD PFS 1 and 5 years	31% 4%, 27% 71 and 48%	NA	Prolonged responses even after treatment discontinuation
Blay JY et al. (144)	2022	TSGCT	II	Vimseltinib 30 mg twice weekly	57 (46 cohort A, 11 cohort B)	45 (21–71)	Safety RR CB (PR+SD)	49% cohort A 44% cohort B CB 100%	NA	Manageable toxicity, effective

(Continued)

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TABLE 3 Continued

Authors/ Study [ref]	Year reported	Diagnosis	Phase	Drug	Number of patients	Median age, years (range)	Endpoints	Outcome	p	Key points
MOTION trial NCT05059262	ongoing	TSGCT	III	Vimseltinib 30 mg twice a week	Ongoing	18 yo or older	ORR at 25 weeks ORR per tumor volume score PROs	Ongoing	–	Ongoing

R, retrospective; PFS, progression free survival; RR, response rate; ORR, overall response rate; CR, complete response; PR, partial response; SD, stable disease; CBR, clinical benefit rate; RECIST, Response Evaluation Criteria in Solid Tumors; DOR, duration of response; LT, long term; NA, not available.

outcomes and detriment to quality of life. Treatment paradigms still vary, given the rarity of these diseases and lack of consensus guidelines. Misdiagnoses are frequent and contribute to suboptimal management, worse outcome, and inadequate patient experience (2, 177, 178). All this is being improved thanks to *ad-hoc* instituted working groups and joint effort of scientists and patient associations across the world. For example, a global consensus meeting held in 2018 brought together world experts and started the process of defining a standard of care for DF. Practice changing conclusions were reached such as the recommendation to proceed with a period of active surveillance for newly diagnosed DF and to consider medical treatment as first option rather than surgery (9). Prospectively controlled clinical trials require partnership and are critical to validating future treatment recommendations for these and other locally aggressive mesenchymal neoplasms. Many efforts have been made in the past few years to identify prognostic and predictive biomarkers and select the best candidates and potential responders to treatment. Recently published studies have significantly contributed to the understanding of the natural history and potential prognostic significance of mutational status in DF. Although no association reached statistical significance, a trend toward worst outcome for tumors harboring mutations involving codon 45F of the CTNNB1 gene, for APC mutated DF and for non-extremity site of disease was uncovered (19, 77, 79). There are not known prognostic factors for GCT or TSGC that can help stratify patients. Massive parallel sequencing of 34 resection specimens of TSGCT detected the presence of a CBL missense mutation in 35% of tumors which was significantly associated with shorter time to local recurrence (179).

Complexity is added to the management of locally aggressive mesenchymal tumors by the unsatisfactory correlation between RECIST assessment and treatment effectiveness. As postulated by many, a better surrogate of treatment efficacy may be the change of T2 signal on MRI; a shift from long to short T2 signal has been in fact observed in DF when tumor histology transitioned from more cellular to more fibrous, hypocellular tissue (180). Similarly, for GCT, RECIST assessment is not an accurate measure of treatment efficacy and the use of modified PET scan criteria or inverse Choi density/size criteria have been proposed to assess response to denosumab (181). Comparable limitations challenge response assessment for TSGCT for which a volumetric comparison of the tumor pre and post treatment may represent a more precise way of measurement than diameter comparison, given the irregular shape of the tumor (6).

Finally, how to select the patients that may benefit the most from treatment and for how long to treat are crucial points that need to be addressed. The newly introduced drugs have shed some light, but they have also uncovered the very specific challenge of exposing patients with non-malignant conditions to the risk of potential long-term toxicity. Many aspects that go beyond the disease itself warrant careful consideration. These patients report persistent pain, emotional distress, and financial hardship (182, 183). While these are non-malignant diseases, the long-term effects of treatments and impact on quality of life resemble cancer in many ways. Patient reported outcomes are a necessary tool to finally strike a balance between the desirable disease control and other non-

negotiable aspects such as family planning, ability to work, financial wellness, and good overall quality of life (8, 172, 173). Given the rarity of this class of tumors, complex patient needs, and to avoid suboptimal outcomes, treatment planning should be individualized and planned in the context of an expert multidisciplinary team.

Author contributions

AM drafting, writing, editing the manuscript, collecting and analyzing data. JZ collecting and analysing data. MC data collection, editing the manuscript. BW collecting and analysing data, editing the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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