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Nádorové mikroprostředí sarkomů měkkých tkání a jeho prediktivní význam v moderní onkologické léčbě

Tumor microenvironment of soft tissue sarcomas and it's predictive significance in modern oncological treatment

Disertační práce

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V Praze 5. 1. 2023

MUDr. Andrej Ozaniak

**SEZNAM ZKRATEK**

| | |
|---|---|
| CT | výpočetní tomografie |
| DDLPS | dediferencovaný liposarkom |
| FAP | familiární adenomatozní polypóza |
| FNCLCC | Fédération Nationale des Centres de Lutte Contre Le Cancer |
| GIST | gastrointestinální stromální tumor |
| HIV | Human Immunodeficiency Virus |
| ILP | izolovaná končetinová perfuze |
| ITK | inhibitory thyrozin kináz |
| LMS | leiomyosarkom |
| LPS | liposarkom |
| MLPS | myxoidní liposarkom |
| MPLPS | myxoidní pleomorfní liposarkom |
| MPNST | maligní nádor z pochev periferních nervů |
| MR | magnetická rezonance |
| NK buňky | natural killer buňky |
| PLPS | pleomorfní liposarkom |
| RAAS | radiačně asociovaný angiosarkom |
| SFT | solitární fibrózní tumor |
| STS | soft tissue sarcoma |
| TILs | infiltrující lymfocyty |
| TNF-α | tumor nekrotizující faktor alpha |
| TSA | tumor-specifické antigeny |
| UPC | nediferencovaný pleomorfní sarkom |
| WDLPS | dobře diferencovaný liposarkom |
| WHO | World Health Organisation |

**OBSAH**

**Abstrakt**

Sarkomy (Soft tissue sarcomas/STSs) jsou maligní nádory mezenchymálního původu, vyznačující se extrémní heterogenitou v histologické skladbě, biologickém chování i klinickém projevu. Většina se vyznačuje chemorezistencí a radiorezistencí. Klíčovým prognostickým faktorem pro celkové přežívání a riziko vzdálených metastáz je histopatologický stupeň diferenciace neboli grade nádoru. Grade ovšem nemá vliv na riziko lokální recidivy. Radikální chirurgický zákrok je často jedinou možnou léčebnou modalitou nebo minimálně hraje hlavní roli v terapii. U generalizovaného onemocnění jsou možnosti léčby značně omezené. Chemosenzitivita STSs je všeobecně velmi nízká, s výjimkou některých méně častých podtypů, a celkově činí jenom 5–10 %. Radioterapie je v mnoha případech standardní součástí léčebného protokolu. Podává se zpravidla v neoadjuvantním nebo adjuvantním podání, ovšem její využití u generalizovaných pacientů je okrajové a nezlepšuje prognózu.

Protinádorová imunoterapie je terapeutická modalita, která využívá fyziologických schopností buněk imunitního systému v boji proti nádorovému onemocnění. Není tedy založena na cílené eliminaci rychle proliferujících nádorových buněk, nýbrž na stimulaci imunitních buněk za účelem likvidace nádorových antigenů. V oblasti imunoterapie měkkotkáňových sarkomů bylo testováno nespočet různých strategií, avšak na rozdíl od jiných solidních tumorů jako například maligního melanomu či karcinomu ledviny, nevedly u sarkomů tyto přístupy k signifikantní regresi nádorové hmoty. Jedním z hlavních důvodů je obrovská histologická heterogenita těchto nádorů a různorodá infiltrace sarkomů imunitními buňkami.

Cílem tohoto výzkumného projektu byla prospektivní a retrospektivní analýza nádorových vzorků u pacientů s měkkotkáňovým sarkomem, detailní popis

nádorového mikroprostředí měkkotkáňových sarkomů a identifikace vhodných terapií pro pacienty s metastatickým onemocněním.

V rámci projektu jsme pracovali s vzácnými kohortami pacientů, které jsme dále dělili dle jejich histologie a dalších klinicko-patologických charakteristik. Prostřednictvím analýzy klinických vzorků, imunohistochemie, průtokové cytometrie a multiplexových metodik jsme vyhodnocovali nejen fenotypické a funkční charakteristiky imunitních buněk v měkkotkáňových sarkomech, ale hodnotili jsme též faktory, které ovlivňují četnost chirurgických komplikací, riziko recidivy či progresi onemocnění.

V naší první observační studii, která byla zaměřena pouze na histologickou entitu solitární fibrózní tumor, jsme pozorovali vysokou frekvenci mylných primárních diagnóz a vliv nádorové lokalizace na rozvoj metastáz či recidivu onemocnění. Definovali jsme faktory, které souvisely s pooperačními komplikacemi, a rozebrali úskalí diagnostiky a léčby solitárních fibrózních tumorů.

V další studii, kterou byl přehledový článek zabývající se nádorovým mikroprostředím měkkotkáňových sarkomů, jsme diskutovali všechna dostupná data týkající se role T-buněk v nádorovém mikroprostředí sarkomů měkkých tkání. Zaměřili jsme se mimo jiné i na další hojně se vyskytující imunitní buňky v nádorovém mikroprostředí a probírali jejich prognostickou a prediktivní roli. V této práci jsme prezentovali všechny klinické studie zaměřené stimulaci imunitního systému prostřednictvím cílení konkrétní buněčné subpopulace u sarkomů měkkých tkání. Tyto studie jsme následně diskutovali z hlediska racionality přístupu a možných kombinací jednotlivých terapeutik.

V rámci našeho nejvýznamnějšího projektu jsme sledovali potenciální aditivní účinky kombinace anti-PD-1 a anti-CD47 imunoterapií v léčbě sarkomů měkkých tkání.

Předpokládali jsme, že in vitro se budou účinky obou terapií potencovat, neboť nádorové mikroprostředí měkkotkáňových sarkomů má vysoké zastoupení makrofágů a druhou nejpočetnější populací imunitních buněk jsou T-buňky. Do kohorty jsme zařadili 66 pacientů, kterým byla nejprve stanovena exprese molekuly CD47 v nádorovém mikroprostředí. Následně byly vybraným pacientům izolovány tumor-infiltrující lymfocyty z nádorové tkáně a získané buněčné suspenze byly *in vitro* kultivovány za přítomnosti kostimulačních molekul, anti-PD-1 monoklonální protilátky (nivolumab), anti-CD47 monoklonální protilátky, a též za přítomnosti obou těchto terapeutik. Funkce buněk byla hodnocena prostřednictvím detekce cytokinů metodou Luminex. Zatímco u vybraných diagnóz byla *in vitro* účinnost jednotlivých terapií vyjádřena vysokou mírou aktivace imunitních buněk, kombinované podání obou terapeutik způsobilo zásadní útlum buněk a sníženou produkci prozánětlivých cytokinů.

Naše práce napomohly pochopení role nádorového mikroprostředí v prognóze pacientů s měkkotkáňovým sarkomem, definovaly faktory spojené s biologickým chováním nádorů a představily moderní přístupy, jimiž lze nádorové mikroprostředí analyzovat a hodnotit efekt imunoterapie.

**Klíčová slova:** sarkom měkké tkáně, imunoterapie, nádorové mikroprostředí, kombinovaná léčba, solitární fibrózní tumor, T-buňky, PD-1, CD47.

**Abstract**

Soft tissue sarcomas (STSs) are malignant tumors of mesenchymal origin, characterized by an extreme heterogeneity in histological composition, biological behavior, and clinical manifestation. Most STSs are chemo- and radiotherapy resistant. A key prognostic factor predicting the risk of distant metastases and affecting the overall survival is the tumor grade. However, grade has not been associated with the risk of local recurrence. Radical surgical procedure is in many cases the only possible treatment modality or at least plays a main role in the multimodal treatment. For patients with distant metastases, the treatment options are very limited. The chemosensitivity of STSs is generally very low, with the exception of several less common subtypes, and accounts for only 5–10% of the cases. In many cases, radiotherapy is a standard part of the treatment protocol. It is usually given in either neoadjuvant or adjuvant settings. However, radiotherapy administration in generalized patients does not improve the prognosis.

Cancer immunotherapy is a therapeutic modality that utilizes the physiological cytotoxic antitumoral abilities of the immune cells. Therefore, it does not target the rapidly proliferating tumor cells but rather stimulates the immune cells. A wide variety of different strategies have been tested in the treatment of soft tissue sarcomas, but unlike in other solid tumors, such as malignant melanoma or kidney cancer, these approaches did not lead to significant regression of the tumor mass in sarcomas. One of the main reasons is the enormous histological heterogeneity of these tumors and thus, diverse infiltration of STSs with the immune cells.

The aim of this research was to conduct a prospective and retrospective analysis of tumor samples from patients with soft tissue sarcoma. We also aimed to describe the tumor microenvironment of STSs and identify possible treatment

modalities for patients with metastatic disease. We created and closely monitored our study cohort, which was divided into subgroups according to diverse criteria based on the tumor histology, the immune infiltration, the grade (low-grade/high-grade), and the stage of the disease. Formalin-fixed and paraffin-embedded (FFPE) tissue blocks were retrospectively obtained from patients and used for the immunohistochemical evaluation of immune infiltrates, including CD8$^+$ T-cells, CD4$^+$ T-cells, macrophages, and others immune cells in the tumors. Through analysis of the clinical specimens, immunohistochemistry, flow cytometry and multiplex methodology, we evaluated not only the phenotypes and functional characteristics of immune cells in sarcomas, but also factors affecting surgical complications, risk of local recurrence or distant spread.

In our first observational study that was focused on solitary fibrous tumors, we identified high rate of primary misdiagnoses and defined the role of primary tumor location on the development distant metastases or local recurrence of the disease. We reported several factors that were associated with postoperative complications and we discussed the pitfalls of diagnostics and treatment of solitary fibrous tumors.

In our review article, we further focused on the immune infiltrates in the tumor microenvironment of soft tissue sarcomas and we discussed all available data regarding the role of T-cells in the tumor microenvironment of soft tissue sarcomas. We also described other immune cells and we discussed their prognostic and predictive role. In this article, we presented all promising clinical trials focused on the stimulation of the immune system by targeting specific cell subpopulations in soft tissue sarcomas. Together we then discussed rational approaches and potential combinations of certain therapeutics.

In our key project, we were evaluating potential additive effects of anti-PD-1 and anti-CD47 combination therapy in soft tissue sarcomas. Given the fact that the TME of

soft tissue sarcomas in predominantly infiltrated by macrophages and T-cells, we hypothesized that a combination of both therapies may represent a particularly relevant approach. In this cohort we selected 66 patients. In this cohort study, 66 patients who underwent surgery were enrolled. Tumor cells and tumor-infiltrating immune cells were analyzed using flow cytometry and immunohistochemistry. In cell suspensions obtained from surgical resections, human T-cells were activated by superparamagnetic polymer beads and cultured in the absence or presence of therapeutic monoclonal antibodies (anti-PD-1, anti-CD47, and anti-PD-1 + anti-CD47). Supernatants from cell suspensions were analyzed using multiplex Luminex cytokine bead-based immunoassays. In selected histological subtypes, the efficacy of individual therapies was reflected in the high degree of activation of the immune cells, however to our surprise, co-administration of both therapies significantly decreased the production of proinflammatory cytokines.

Our work contributed to the understanding of the tumor microenvironment in soft tissue sarcomas and its role in patients' prognosis. Furthermore, we defined factors associated with biological behaviour of the tumors and introduced modern therapeutic approaches to analyse the tumor microenvironment and evaluate the effect of immunotherapy.

**Keywords:** soft tissue sarcoma, immunotherapy, tumor microenvironment, combined treatment, solitary fibrous tumor, T-cells, PD-1, CD47.

# 1   ÚVOD

## 1.1   Sarkomy měkkých tkání

### 1.1.1   Epidemiologie a základní charakteristika

Sarkomy jsou extrémně heterogenní skupinou maligních onemocnění, u nichž maligní nádorová populace vychází z embryonální mezenchymální tkáně, která dává vzniknout pojivové tkáni, jako je vazivo, chrupavka a kost, a dále cévám, svalům a nervům. Sarkomy představují jenom přibližně 1 % všech malignit v dospělé populaci. Celá skupina se dělí na dvě základní podskupiny – kostní sarkomy reprezentující přibližně 10 % sarkomů a sarkomy měkkých tkání (angl. soft tissue sarcoma, zkr. STS), které tvoří přibližně 90 % sarkomů. Nejčastějšími kostními sarkomy jsou osteosarkom, chondrosarkom a Ewingův sarkom. Pojem STS v současné době v sobě zahrnuje dle WHO klasifikace více než 80 histologických entit [1]. Ve všeobecnosti se jedná o extrémně vzácná maligní nádorová onemocnění, jejichž incidence jako celku se pohybuje kolem 6,4/100 000 případů ročně [2]. Navíc i incidence benigních mezenchymálních nádorů je stonásobně vyšší než sarkomů. Základní histologické skupiny měkkotkáňových nádorů zobrazuje **Tabulka č. 1.**

| Histologické skupiny |
|---|
| Adipocytární tumory |
| Fibroblastické a myofibroblastické tumory |
| Fibrohistiocytární tumory |
| Vaskulární tumory |
| Perivaskulární tumory |
| Tumory hladké svaloviny |
| Tumory kosterní svaloviny |
| Gastrointestinální stromální tumory |
| Chondro-oseální tumory |
| Tumory z pochev periferních nervů |
| Tumory nejisté diferenciace |
| Nediferencované malé kulatobuněčné sarkomy |

**Tabulka č. 1. Základní histologické skupiny**

*Tabulka znázorňující základní dělení měkkotkáňových nádorů dle histologie*.

Každá histologická skupina v sobě zahrnuje čtyři biologicky rozdílné podtypy nádorů, které jsou znázorněny v **Tabulce č. 2.**

| Dělení histologických skupin | | | |
|---|---|---|---|
| **Benigní** | **Intermediární, lokálně agresivní** | **Intermediární, raritně metastazující** | **Maligní** |
| Lokálně nedestruktivní růst | Lokálně destruktivní růst | Lokálně destruktivní růst | Lokálně destruktivní růst |
| Nízké riziko lokální recidivy | Vysoké riziko lokální recidivy | Riziko lokální recidivy | Vysoké riziko lokální recidivy |
| Nemetastazující | Nemetastazující | Nízký potenciál metastazování (< 2 %) | Vysoké riziko metastazování |

**Tabulka č. 2. Dělení nádorů dle biologického chování**

*Tabulka znázorňující hlavní kategorie nádorů dle biologického chování. Toto dělení zohledňuje charakter prorůstání tumoru, riziko lokálních recidiv a pravděpodobnost metastazování.*

Histologický grading STS se provádí na základě doporučení francouzské organizace Fédération Nationale des Centres de Lutte Contre Le Cancer/FNCLCC (**Tabulka č. 3**). V rámci této klasifikace jsou hodnoceny tři parametry: diferenciace tumoru, počet mitóz a nekróza tumoru. Tumory na základě toho rozděluje na grade 1, 2, 3.

| Histologický grading podle klasifikace FNCLCC | |
|---|---|
| **Diferenciace tumoru** | |
| Skóre 1 | Výrazně se přibližující normální tkáni |
| Skóre 2 | Histologická typizace je jistá |
| Skóre 3 | Embryonální nebo nediferencované sarkomy |
| **Počet mitóz (na 1,7 mm$^2$)** | |
| Skóre 1 | 0–9 mitóz |
| Skóre 2 | 10–19 mitóz |
| Skóre 3 | > 19 mitóz |
| **Nekrózy v tumoru** | |
| Skóre 0 | Bez nekróz |
| Skóre 1 | < 50 % nekróz |
| Skóre 2 | ≥ 50 % nekróz |
| **Histologický grade** | |
| | Grade 1: totální skóre 2, 3 |
| | Grade 2: totální skóre 4, 5 |
| | Grade 3: totální skóre 6, 7, 8 |

**Tabulka č. 3. Grading nádoru na základě Fédération Nationale des Centres de Lutte Contre Le Cancer (FNCLCC)**

*Tabulka znázorňující jednotlivé parametry, na jejichž základě je dle FNCLCC určován histologický grade nádoru. Histologický grade zohledňuje počet mitóz, intratumorální nekrózy a stupeň diferenciace. Součet bodů v jednotlivých parametrech dá výsledný grade.*

Grade nádoru je nejdůležitější prognostický faktor [3]. Mezi další důležité prognostické faktory patří velikost tumoru a jeho lokalizace, resekabilita tumoru,

přítomnost vzdálených metastáz, kvalita chirurgických resekčních okrajů a předoperační/perioperační ruptura nádoru. Tumory lokalizované v retroperitoneu mají obecně nejhorší prognózu [4]. Současně platí, že pacienti starší 65 let mají horší prognózu než mladší [5, 6].

Jak je znázorněno na **Obrázku č. 1**, mezi nejčastěji se vyskytující histologické podtypy STS řadíme skupinu liposarkomů (dobře diferencovaný liposarkom (WDLPS), dediferencovaný liposarkom (DDLPS), myxoidní liposarkom (MLPS), pleomorfní liposarkom (PLPS) a myxoidní pleomorfní liposarkom (MPLPS), dále pak leiomyosarkomy (LMS), nediferencované pleomorfní sarkomy (UPS) a GISTy [1].
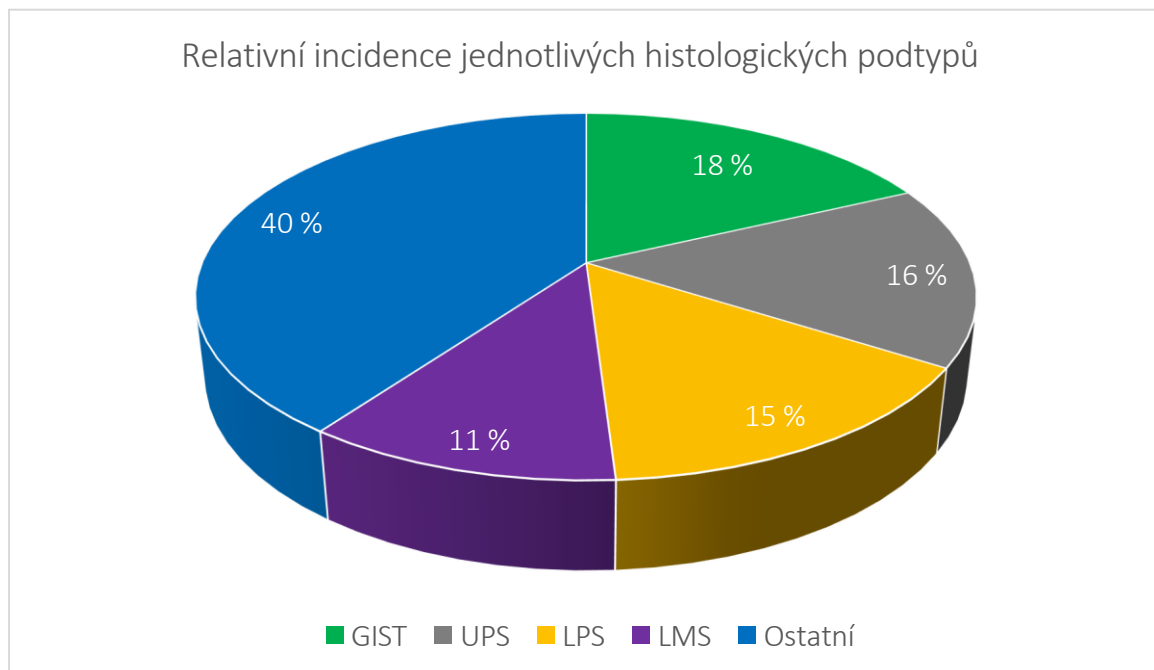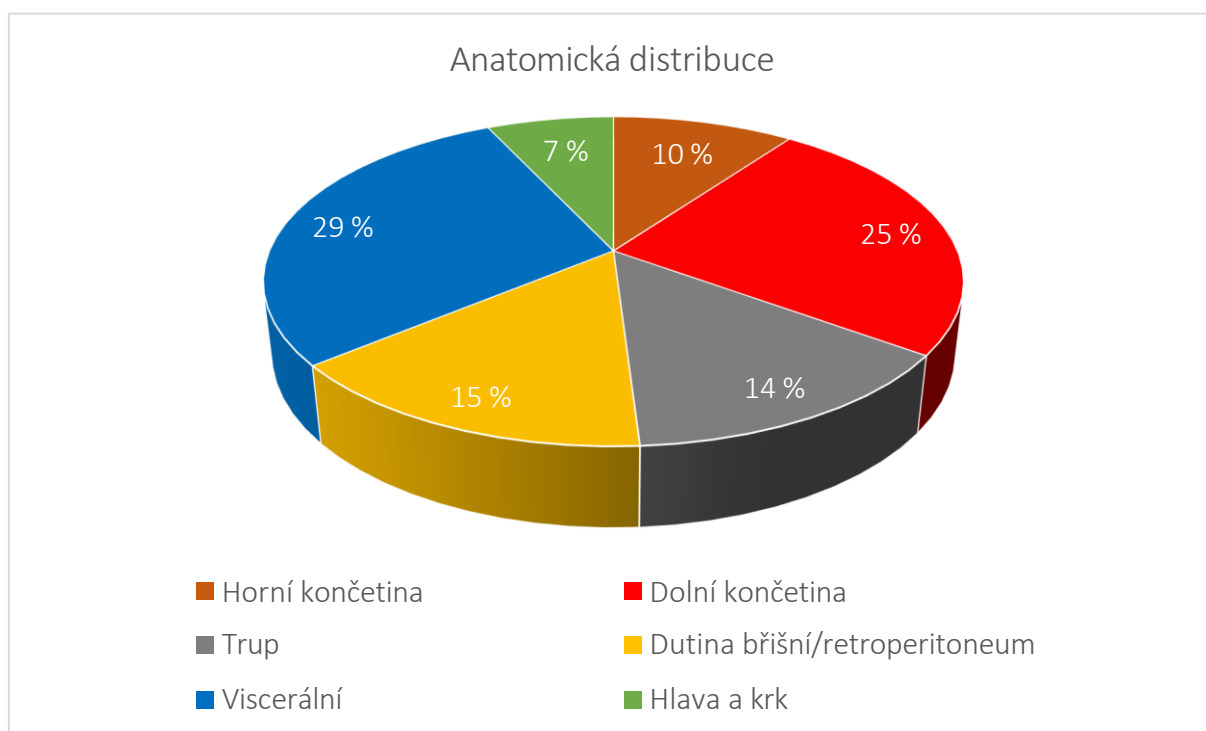


**Obrázek č. 1. Nejčastější histologické typy měkkotkáňových sarkomů**

*Obrázek znázorňující relativní incidenci, tedy výskyt jednotlivých histologických typů v celkovém počtu měkkotkáňových sarkomů bez ohledu na absolutní počty osob v těchto skupinách. Zkratky: GIST: gastrointestinální stromální tumor, UPS: nediferencovaný pleomorfní sarkom, LPS: liposarkom, LMS: leiomyosarkom.*

Tyto čtyři skupiny nejčastěji se vyskytujících STS se vyznačují incidencí < 1/100 000/rok. Incidence většiny zbylých sarkomů, které souhrnně označujeme jako raritní sarkomy, je < 2/1 000 000/ rok. Výskyt STS je většinou sporadický. Ovšem dobře dokumentovaný je i jejich výskyt v rámci hereditárních syndromů – desmoid/agresivní fibromatóza u pacientů s familiární adenomatozní polypózou (FAP) [7], GIST a maligní nádor z pochev periferních nervů (MPNST) u pacientů s neurofibromatózou [8] nebo sarkomy u pacientů s Li-Fraumeniho syndromem [9]. Vzácně jsou sarkomy také asociované s předešlou iradiací [10], virovou infekcí nebo imunodeficiencí, jako je například Kaposiho sarkom u pacientů s HIV [11]. Vztah k radiačnímu ozáření můžeme sledovat typicky u pacientek po radioterapii pro karcinom prsu. Takový sarkom se pak nazývá zpravidla radiačně asociovaný angiosarkom (RAAS).

STS se mohou vyskytovat prakticky kdekoliv na těle. Většinou se manifestují jako nebolestivá rezistence různé velikosti. Mohou být povrchové, uložené epifasciálně, nebo v hloubce uložené, subfasciálně. Anatomickou distribuci zobrazuje **Obrázek č. 2.**

**Anatomická distribuce**

- Horní končetina
- Dolní končetina
- Trup
- Dutina břišní/retroperitoneum
- Viscerální
- Hlava a krk

**Obrázek č. 2. Typické lokalizace sarkomů v těle**

*Obrázek znázorňující četnost výskytu měkkotkáňových sarkomů na základě typické anatomické lokalizace.*

### 1.1.2   Diagnostika a léčba sarkomů měkkých tkání

Každá měkkotkáňová povrchová léze větší než 5 centimetrů nebo hluboce uložená jakékoliv velikosti by měla být referovaná do center specializujících se na problematiku sarkomů [12]. Jak je znázorněno na **Obrázku č. 3,** byl prokázán signifikantní rozdíl v celkovém přežití a přežití bez lokální recidivy, pokud jsou pacienti referováni do sarkomových center nebo pokud jejich léčba probíhá mimo tato centra,
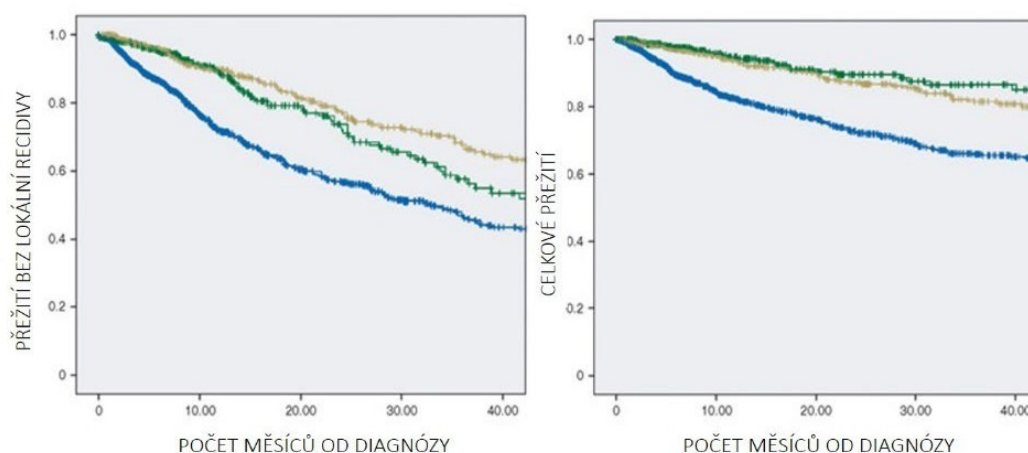


**Obrázek č. 3. Vliv centralizace pacientů na celkové přežití pacientů a riziko lokální recidivy**

*Záznam přežívání pacientů bez lokální recidivy (vlevo) a celkového přežívání (vpravo) pacientů operovaných mimo sarkomová centra (modrá křivka), operovaných v sarkomových centrech s méně než 10 operacemi sarkomů ročně (zelená křivka) a s více než 10 operacemi sarkomů ročně (hnědá křivka). Převzato z Annals of Oncology, Dec 1019, Vol 30, Issue 12, str. 2008–2009.*

Centralizace je proto první krok ke správnému managementu těchto nemocných [13]. Mezi rizikové faktory, které jsou často spojované se sarkomem, patří subfasciální

uložení, velikost větší než 5 centimetrů a velikostní progrese. Hluboce uložené měkkotkáňové nádory bývají dlouhodobě asymptomatické, což vede k jejich růstu do enormních rozměrů. Typická, zejména pro retroperitoneální sarkomy, je velikost nad 20 centimetrů v průměru v době diagnózy. Diagnostický algoritmus zobrazuje **Obrázek č. 4.**
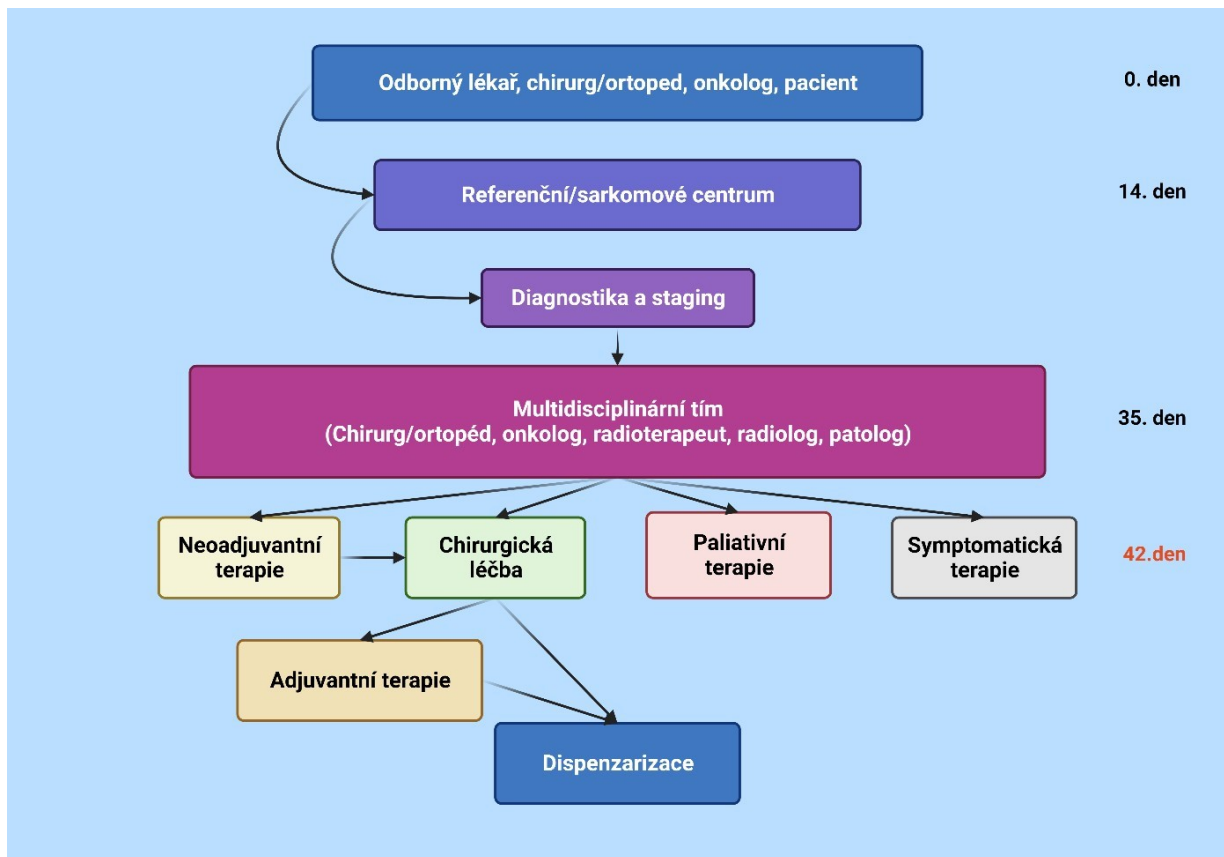


**Obrázek č. 4. Diagnostický algoritmus**

*Obrázek znázorňující optimální časové intervaly v rámci diagnostického procesu od suspekce až po podání první linie terapie. Obrázek byl vytvořen s využitím softwaru BioRender (ref.č. XB24XJQM4D).*

Po zhodnocení lokálního nálezu musí klinik rozhodnout ohledně způsobu odběru biopsie a dokončit kompletní staging [14, 15]. Jenom výjimečně se souhlasem multidisciplinárního týmu nemusí být biopsie provedena. Pro lokální zobrazení je

nejlepší modalitou magnetická rezonance (MRi) především pro končetinové sarkomy, sarkomy trupu nebo výpočetní tomografie (CT) především pro sarkomy retroperitonea a dutiny břišní, a viscerální sarkomy. V rámci stagingu se provádí standardně CT hrudníku k vyloučení generalizace. Plíce jsou nejčastějším místem generalizace. Postižení lokoregionálních lymfatických uzlin je zcela výjimečné (< 1%). Zobrazení a zhodnocení lymfatických uzlin je ovšem nutné u epiteloidního sarkomu, světlo-buněčného sarkomu, synoviálního sarkomu a angiosarkomu. CT břicha v rámci stagingu je indikované zejména u MLPS a LMS. U angiosarkomu doplňujeme i CT mozku k vyloučení mozkových metastáz [16].

Základem diagnostiky je histopatologické vyšetření bioptických vzorků nádoru v rukách zkušeného patologa, specializujícího se na problematiku měkkotkáňových nádorů [17]. Součástí vyšetření je morfologie doplněná o imunohistochemické a často také molekulární metody [18]. Po stanovení histologie a kompletním stagingu je pacient referován na multidisciplinárním setkání, kde je rozhodnuto o adekvátní nejlepší první linii léčby, eventuálně o symptomatické terapii. Multidisciplinární tým pozůstává z členů specializovaných v těchto oborech: zobrazovací metody a intervenční radiologie, patologie, onkologie, radioterapie, onkochirurgie [13]. Léčba pacientů s STS je ve většině případů multimodální. Kromě histologického podtypu a stadia nemoci zasahuje do způsobu léčby i primární lokalizace sarkomu a rozsah plánované resekce.

### 1.1.2.1 Léčba lokalizovaných primárně resekabilních končetinových sarkomů a sarkomů trupu

Chirurgická resekce je základní modalitou léčby. Prováděna by měla být onkochirurgem specializovaným v problematice STS. Základním cílem je provedení en-bloc excize s negativním mikroskopickým okrajem (R0). Ideálním způsobem k docílení toho je resekce tumoru s lemem zdravé tkáně. O šířce lemu rozhoduje několik faktorů – histologický typ nádoru, možnost neo/adjuvantní radioterapie, vzdálenost kritických struktur, přítomnost přirozených bariér (svalová fascie, vaskulární adventicie, periost, epineurium) [19]. Dle rozsahu rozlišujeme čtyři typy resekčních výkonů: 1. intralesionální resekci, 2. marginální resekci, 3. širokou resekci, 4. kompartmentovou resekci. Ideální rozsah chirurgického výkonu je široká resekce nebo kompartmentová resekce. Nikdy bychom neměli provádět intralesionální resekci, k marginální resekci většinou dochází v důsledku nerespektování základních diagnosticko-terapeutických postupů. V indikovaných případech i marginální resekce může být indikovaná na podkladě rozhodnutí multidisciplinárního týmu v případě nutnosti prezervace kritických anatomických struktur [20-22]. V tomto případě je nutné v rámci týmu diskutovat využití neoadjuvantních modalit léčby [23].

Radioterapie je základní součástí léčby high-grade sarkomů [24]. Může být podána v neoadjuvantním nebo adjuvantním schématu. Lokální rekurence ani celkové přežívání není ovlivněno timingem radioterapie, ovšem neoadjuvantní podání má několik výhod: nižší dlouhodobou morbiditu, možnost ochrany kritických anatomických struktur před resekcí, snížení rizika R1 resekce [25, 26]. Naopak je pozorován vyšší výskyt časných ranních komplikací [27]. V neoadjuvantním provedení se podává celková dávka 50 Gy rozdělená v 1.8–2.0 Gy frakcích. V adjuvantním provedení se podávají dávky do 66 Gy [16]. Radioterapie nemusí být podána v případě skutečně

kompartmentových resekcí, při kterých je tumor kompletně odstraněn i se svalovou skupinou, ze které vyrůstá. Myxoidní liposarkom (MLPS), myxofibrosarkom, solitární fibrózní tumor (SFT) a extraskeletální myxoidní chondrosarkom mají výbornou radiosenzitivitu [28-30].

Na podání chemoterapie v současné době neexistuje konsenzus mezi jednotlivými centry. Na místě je podání chemoterapie, preferenčně v neoadjuvantním podání u „high-risk" pacientů [31, 32]. To jsou pacienti, jejichž pravděpodobnost desetiletého přežití je < 60%.

U končetinových sarkomů je možností také podání izolované končetinové perfuze (ILP). Ta zlepšuje lokální operabilitu, ovšem nemá vliv na celkové přežívání [33]. Indikovaná by proto měla být u lokálně inoperabilních nálezů, hrozící neradikální resekce nebo v případě hrozící amputace. V současné době je s využitím neoadjuvantních metod až 95 % zákroků prováděných jako takzvané končetinu záchovné výkony = limb sparing [34].

### 1.1.2.2 Léčba lokalizovaných primárně neresekabilních končetinových sarkomů a sarkomů trupu

V případě primárně neresekabilních nálezů je na místě aplikace neoadjuvantních modalit léčby. Primární chirurgický výkon nemá opodstatnění, má špatné výsledky a nijak pacientovi nezlepšuje prognózu. Cílem neoadjuvantních modalit je zmenšení objemu nádoru a umožnění operability. Možností se naskýtá několik – radioterapie, chemoterapie, konkomitantní chemoradioterapie nebo izolovaná končetinová perfuze s tumor nekrotizujícím faktorem alpha (TNF-α) s melphalanem nebo regionální hypertermie v kombinaci s chemoterapií [16]. Pokud neoadjuvantní terapie vede ke zlepšení lokální operability s vizí R0 nebo R1 resekce, indikované je operační řešení. Pokud neoadjuvantní terapie nevede ke zlepšení lokální operability, jsou pacienti indikovaní k paliativní systémové léčbě.

### 1.1.2.3 Léčba pokročilých/generalizovaných primárně resekabilních sarkomů

Způsob léčby generalizovaných pacientů primárně resekabilních je výsledkem konsensu multidisciplinárního týmu. O způsobu léčby rozhoduje rozsah metastatického postižení. Pacienti s extrapulmonálním metastatickým postižením jsou indikovaní k paliativní systémové terapii [16]. V případě pacientů s izolovanou generalizací do plic jsou možnosti léčby znatelně rozmanitější. V případě „nevelkého" metastatického postižení, kdy je možné veškerou nemoc chirurgicky odstranit, mohou být pacienti indikovaní k operačnímu výkonu [35]. U „high-risk" pacientů je na místě podání neoadjuvantní chemoterapie k posouzení biologie základního onemocnění.

### 1.1.2.4 Léčba pokročilých/generalizovaných neresekabilních sarkomů

Pacienti s metachronními plicními metastázami (disease-free interval > 1 rok), pokud jsou resekabilní, s vyloučeným mimoplicním onemocněním, jsou indikovaní k plicní metastazektomii. Preferované jsou minimálně invazivní parenchym šetřící resekční výkony.

Základem léčby pokročilých/generalizovaných neresekabilních sarkomů je systémová chemoterapie. V první linii léčby se využívá antracyklinu v monoterapii nebo kombinované režimy, zejména s ifosfamidem [36, 37].

U LMS nebo SFT se v rámci kombinovaných režimů využívá doxorubicin s dacarbazinem [38, 39].

Angiosarkomy bývají vysoce senzitivní k podání taxanů [40].

Pacienti s pokročilým dermatofibrosarcoma protuberans jsou v první linii léčby léčeni inhibitory thyrozin kináz (ITK), konkrétně Imatinibem [41].

Pacienti s prokázaným NTRK-rearranged sarkomem jsou léčeni pomocí NTRK-inhibitorů – larotrectinibu, entrectinibu [42]. Jejich podání je indikované i v rámci neoadjuvance s cílem zlepšení lokální operability.

## 1.1.2.5 Léčba retroperitoneálních sarkomů

Problematika retroperitoneálních sarkomů zasluhuje zvláštní rozbor. S celosvětovou incidencí 0,5/100 000 připadá na Českou republiku jenom kolem 50 případů ročně [43]. Zároveň je obecně známo, že sarkomy retroperitonea mají zdaleka nejhorší výsledky. Je to dáno poměrně dlouhou dobou klinické asymptomatičnosti a dále složitostí anatomických poměrů v retroperitoneu, a tedy složitými možnostmi „radikálního" neboli „bezpečného" chirurgického okraje [44, 45]. Pacienti s retroperitoneální měkkotkáňovou masou jakékoliv velikosti by proto měli být okamžitě referovaní do sarkomových center, zabývajících se problematikou retroperitoneálních tumorů [46-48].

Základním předpokladem pro úspěšnou léčbu je povědomí o tom, s jakým typem sarkomu v retroperitoneu máme co do činění. V prvním kroku je proto nutné provedení bioptické verifikace tumoru pod CT kontrolou. Indikovaný je odběr pomocí core-cut jehly velikosti minimálně 16G a odběr minimálně 6–8 reprezentativních vzorků nádorové tkáně (v případě heterogenity nádoru odběr jak solidních, tak lipomatozních složek) z dorzálního nebo laterálního přístupu, pokud je to možné, k zajištění dostatečného množství materiálu pro imunohistochemické a eventuálně i molekulární vyšetření [49]. Riziko vzniku implantačních metastáz v průběhu punkčního kanálu je minimální a benefity biopsie zcela jednoznačně převyšují potencionální rizika [50, 51]. Provedení laparoskopické nebo otevřené biopsie je přísně kontraindikované, nakolik způsobuje kontaminaci operačního pole, ničí přirozené anatomické vrstvy, a stejně umožňuje odběr jen povrchové části tumoru, takže u tumorů se silným pouzdrem jsou odběry nevalidní. Navíc výrazně snižuje možnost adekvátního a radikálního

chirurgického výkonu. Ke stagingu onemocnění se používá CT hrudníku, břicha a malé pánve [52].

Chirurgická léčba je základní modalitou terapie u sarkomů retroperitonea [53]. Užití neoadjuvantních modalit u primárně resekabilního nádoru v současné době nemá své opodstatnění v datech. Možné je ovšem využití neoadjuvantní radioterapie u dobře diferencovaného liposarkomu a low-grade dediferencovaného liposarkomu [54]. Užití neoadjuvantní chemoterapie je na zvážení kupříkladu u v retroperitoneu vzácně se vyskytujícího synoviálního sarkomu [55]. Další adekvátní možností využití neoadjuvantní terapie je zlepšení lokální operability, jako jsou využití chemoterapie u LMS vycházejícího z vena cava inferior [56], nebo radioterapie u SFT [57].

Rozsah chirurgické resekce je značně ovlivněn histologickým typem sarkomu. Obecně lze ale deklarovat, že cílem výkonu by mělo být en-blok kompletní odstranění tumoru se všemi okolními adherujícími orgány, i pokud tyto nejsou makroskopicky infiltrované [53]. U liposarkomů jsou preferované kompletní exenterace celého retroperitonea, nakolik rozlišení dobře diferencované komponenty od normálního tuku může být obtížné a zavádějící a při podhodnocení situace může vést k odstranění jenom dediferencované části s ponecháním dobře diferencované části nádoru v těle pacienta [58, 59]. Na druhou stranu LMS nebo SFT jsou poměrně dobře odlišitelné od okolní tkáně a u těchto histologických podskupin je na místě zvažování méně radikálních resekčních výkonů [60]. Operační skupinu by proto měl vést chirurg zkušený v problematice sarkomů, na jehož zkušenosti záleží prezervace nebo nutnost resekce orgánů a neuro-vaskulárních struktur [61].

Paliativní debulking má limitovaný benefit pro pacienta s potenciálně výraznými riziky, a proto by měl být indikován přísně selektivně na základě multidisciplinárního rozhodnutí [62].

## 1.2 Role imunitního systému v léčbě nádorů

## 1.2.1 Základy imunitního systému

Imunitní systém je základním mechanizmem sloužícím k udržování homeostázy [63]. Má nezastupitelnou roli v obranyschopnosti před zevními patogeny, zajišťuje autotoleranci vlastních tkání a imunitní dohled nad vnitřními a vnějšími škodlivinami [64]. Součástí imunitního systému jsou primární a sekundární lymfatické orgány [65]. Mezi primární lymfatické orgány patří kostní dřeň a thymus. Jedná se o místa vzniku, diferenciace a vyzrávání imunitních buněk [66]. Sekundární lymfatické orgány jsou místa, kde probíhají hlavní fáze antigenně specifických imunitních reakcí, a patří k nim slezina a lymfatické uzliny [67]. V lidském těle obecně fungují dva imunitní typy imunitních reakcí, které vzájemně kooperují a účastní se obrany proti patogenním mikroorganismům, ale také proti maligně transformovaným buňkám [68]. Jedním z těchto typů imunitních reakcí je reakce složek přirozené imunity, která je fylogeneticky starší, v organizmu je přítomná již při styku s antigenem, reaguje rychle, ovšem nemá schopnost imunologické paměti [69]. Adaptivní imunita, která se aktivuje až po styku s antigenem a postupně vyzrává, reaguje plně až v průběhu několika dní, avšak má schopnost tvorby imunologické paměti, což umožňuje mnohem efektivnější obranu při opakovaném setkání s antigenem [70, 71]. Součástí přirozené imunity jsou buněčné složky a humorální složky. Mezi buněčné složky řadíme fagocytující buňky a přirozené lymfoidní buňky a mezi humorální složky patří komplement, interferony, lektiny a jiné sérové proteiny [69]. Nejdůležitější složkou přirozené imunitní odpovědi v protinádorovém boji jsou takzvané natural killer (NK) buňky, které jsou schopné bez předchozí aktivace či diferenciace cíleně usmrtit nádorovou buňku [72]. Další buňky přirozené imunitní odpovědi, jako jsou například neutrofilní leukocyty či makrofágy,

mohou za určitých okolností pomáhat eliminaci nádoru, jindy však svým působením nádorový růst podporují [73]. Hlavními představiteli adaptivní imunitní odpovědi jsou B-buňky a T-buňky [71]. B-buňky svojí tvorbou protilátek chrání před bakteriemi a extracelulárními patogeny, ovšem mohou se podílet i na patologických stavech, jako jsou například alergické reakce nebo autoimunitní onemocnění [74]. T-buňky představují nejvýznamnější ochranu před viry, intracelulárními patogeny a maligními buňkami [75]. Detailní studium T-buněk odhalilo několik podtříd T-buněk a jejich fenotypické a funkční vlastnosti se mohou značně lišit v závislosti na vyvolávající příčině a spektru produkovaných cytokinů. Tradičně se T-buňky dělí na pomocné, CD4$^+$ T-buňky, a cytotoxické, CD8$^+$ T-buňky [75]. Cytotoxické CD8$^+$ T-buňky jsou v nádorovém mikroprostředí nejdůležitějšími efektorovými buňkami právě díky své schopnosti efektivně eliminovat cílovou buňku prostřednictvím indukce apoptózy či prostřednictvím produkce cytolytických cytokinů. CD4$^+$ T-buňky naproti tomu obsahují i populaci takzvaných T-regulačních buněk, které jsou schopné působit imunosupresivně, a nádorový růst tedy podporují [75, 76]. Na **Obrázku č.5** lze vidět nejčastější populace imunitních buněk nacházející se v nádorovém mikroprostředí.
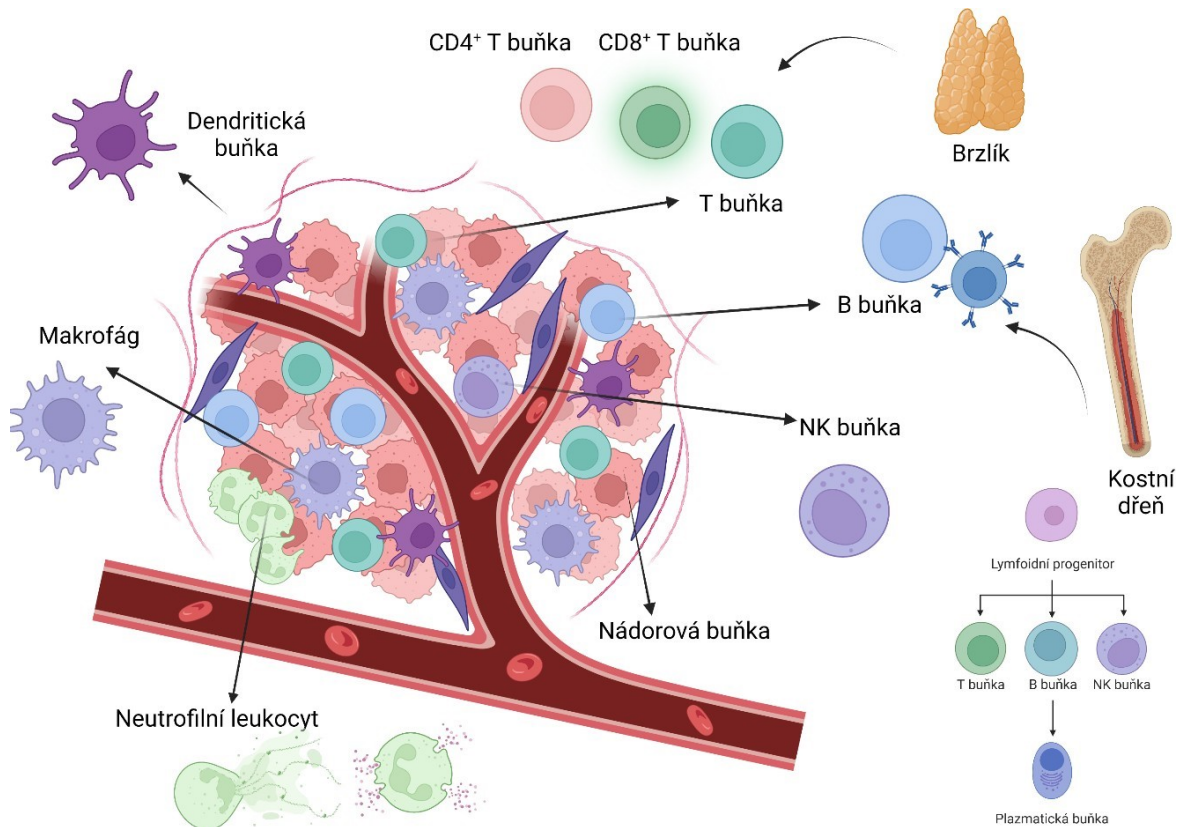
**Obrázek č.5. Nádorové mikroprostředí z hlediska imunitních infiltrátů**

*Zjednodušený model znázorňující schematicky nejčastější populace imunitních buněk nacházejících se v nádorovém mikroprostředí. CD8$^+$ T buňky představují hlavní efektorovou buňku adaptivní imunitní odpovědi, která je schopná po aktivaci prostřednictvím svého povrchového receptoru eliminovat nádorovou buňku. Dendritická buňka kooperuje s CD4$^+$ T buňkou za účelem předávání informací o nádorových antigenech. Ostatní buňky nádorového mikroprostředí se liší svými cytotoxickými schopnostmi a mohou selektivně likvidovat buňky s nízkou expresí MHC I. typu (NK buňky), fagocytovat (makrofágy, neutrofilní leukocyty) či využívat specializovaná granula nebo účinek extracelulárních sítí (neutrofilní leukocyty). Obrázek byl vytvořen s využitím softwaru BioRender (ref.č. WB24XJQD57).*

### 1.2.2 Nádorové mikroprostředí

Nádorové mikroprostředí se tradičně dělí na nádorový parenchym, tvořený maligně transformovanými buňkami vycházejícími z původního orgánu či tkáně, a nádorové stroma, které je tvořené nejen buňkami pojiva, ale též buňkami imunitními [77]. V posledních letech se pozornost výzkumu v protinádorové terapii obrátila právě na buňky stromatu a nyní existují terapie, které cíleně ovlivňují nádorovou angiogenezi, proces metastazování nebo imunitní buňky v nádoru [78]. Pojem tumor-infiltrující lymfocyty (TILs) byl zaveden pro populaci bílých krvinek, která opustila periferní krevní řečiště a vcestovala do nádorového mikroprostředí [79]. Ač je v nádoru celé spektrum imunitních buněk, jako jsou například NK buňky, makrofágy, neutrofilní leukocyty nebo dendritické buňky, pojem TILs je často užíván především pro heterogenní skupinu CD4$^+$ a CD8$^+$ T-buněk, které mají též největší vztah k prognóze pacienta [79].

Korelace dobré prognózy s mírou lymfocytární infiltrace byla jednoznačně zjištěna u karcinomu prsu, plic nebo ovarií [80-82]. Zajímavou výjimku tvoří karcinom ledviny, u něhož se stoupajícím počtem infiltrujících T-buněk byla pozorována horší prognóza, zatímco vysoká infiltrace nádoru NK buňkami byla asociována s dlouhodobějším přežitím [83, 84]. Rozporuplné nálezy v této oblasti zdůrazňují nutnost studia fenotypu a funkce TILs, protože samotné počty nemusejí mít vypovídající hodnotu. U sarkomů měkkých tkání nebylo nádorové mikroprostředí doposud příliš popsáno. Studie jsou limitovány nejen relativně nízkou infiltrací sarkomů imunitními buňkami, ale též vzácností a vysokou histologickou heterogenitou sarkomů, která brání designu velkých observačních studií [85]. Dnes předpokládáme, že nejvýznamnější imunitní populace měkkotkáňových sarkomů jsou T-buňky, NK buňky, makrofágy a T-regulační buňky. Makrofágy byly dokonce označeny několika studiemi za nejpočetnější

skupinu buněk infiltrujících nádorové mikroprostředí u STS [85]. T-buňky v sarkomech mají různý fenotyp a jejich množství se významně liší v závislosti na histologickém typu sarkomu. Například v případě liposarkomů je WDLPS relativně vysoce infiltrován T-buňkami, zatímco MLPS má infiltraci téměř nulovou, a není tedy vhodným kandidátem pro imunoterapii checkpoint inhibitory [86]. Právě zastoupení imunitních infiltrátů v nádorech významně ovlivňuje to, jaký typ imunoterapie lze aplikovat. Dalším aspektem nádorového mikroprostředí je přítomnost tumor-specifických antigenů (TSA) [87]. Je-li definován nějaký TSA, je možné vytvořit imunoterapii cíleně proti danému antigenu. Většina moderních imunoterapií primárně cílí na obnovení efektových funkcí T-buněk [88]. Jsou-li totiž T-buňky dlouhodobě vystaveny stimulaci antigenu, dochází u nich k útlumu efektorových funkcí, který je charakterizován snížením proliferační a cytolytické kapacity a zvýšenou expresí inhibičních molekul, jako jsou například PD1, LAG-3, TIM-3 nebo CTLA-4 [89]. Tento stav a s ním související buněčný fenotyp nazýváme funkční vyčerpání [90]. Vyčerpání T-buněk je velmi typické pro nádorové mikroprostředí, nejedná se ovšem o stav nevratný [90]. Právě imunoterapeutické přístupy cílené na překonání imunologického vyčerpání v nádorovém mikroprostředí jsou nyní první linií léčby celé řady metastatických solidních tumorů [91].

Zatímco role NK buněk a CD8$^+$ T-buněk je široce popisována v kontextu dobré prognózy u pacientů s vysokou infiltrací nádorového mikroprostředí těmito buňkami, makrofágy a jejich role v nádorech jsou značně kontroverzní [92]. Ukázalo se, že vlivem nádorového mikroprostředí může dojít k přesmyku fenotypu makrofágů a ty se stávají pro-tumorigenní [92, 93]. Jejich role pak spočívá především v tvorbě profibrogenních faktorů a tedy pomáhání v tvorbě účinné fibrotické bariéry, která brání nejen vstupu imunitních buněk do nádoru, ale též vstupu imunoterapií [93]. Makrofágy se zdají být nejčetnější populací v STS, ovšem jejich role není zdaleka objasněna [94].

Současně dodnes nejsou v klinických studiích u STS testovány terapie zaměřené na podporu protinádorového působení makrofágů [85].

### 1.2.3 Protinádorová imunoterapie

Imunoterapie nádorových onemocnění se stala revolučním přístupem v léčbě onkologických pacientů [95]. Význam tohoto přístupu je zřejmý i z Nobelovy ceny, která byla udělena v roce 2018 za objev takzvaných checkpoint inhibitorů, které jsou nyní první linií léčby celé řady metastatických nádorů [96]. Imunoterapie má více než stoletou tradici a překvapivě první nádorové onemocnění, které bylo v historii léčeno imunoterapií, byl měkkotkáňový sarkom [97, 98]. První imunoterapeutické přístupy spočívaly v inokulaci streptokoka do místa tumoru a umělém vytvoření erysipelu v této lokalitě. Infekce vedla k masivnímu náboru imunitních buněk do místa sarkomu a u vybraných pacientů došlo k regresi nádorové hmoty [97]. Tento přístup měl bohužel již v době svých počátků spoustu odpůrců a vzhledem k riziku systémové reakce spojené s umělým vyvoláním infekce v místě nádoru došlo postupně k upuštění od tohoto imunoterapeutického přístupu [99]. V dnešní době dělíme imunoterapeutické přístupy dle různých kritérií, z nichž jedním z nich je to, nakolik imunoterapeutický přístup nespecificky stimuluje celý imunitní systém nebo specificky ovlivňuje jednu buněčnou skupinu [100]. Do nespecifických stimulátorů řadíme například BCG vakcínu, která stále představuje významný přístup pro léčbu karcinomu močového měchýře, a dále jsou to například cytokiny, jejichž podávání je nyní vázáno především na kombinace s jinými terapiemi [101, 102]. Předmětem specifických imunoterapií se staly především T-buňky. T-buňky lze ovlivnit přímo aplikací monoklonálních protilátek, které brání jejich funkčnímu útlumu, nebo vyjmutím T-buněk z těla pacienta a jejich ex vivo modifikací [103, 104]. Nejrozšířenějším a technologicky jednodušším přístupem je aplikace monoklonálních protilátek, které se váží na inhibiční receptory T-buněk. Těmto protilátkám se říká checkpoint inhibitory [103]. Checkpoint inhibitory zásadně změnily prognózu pacientů s diagnózami, jako jsou například maligní melanom,

metastatický karcinom plic nebo karcinom ledviny [105-107]. Díky své schopnosti zredukovat i vzdálené metastázy se staly první linií léčby mnoha metastatických nádorových onemocnění, ovšem existuje i několik úskalí, která tuto terapii doprovázejí. Prvním úskalím je omezené množství pacientů, kteří na tuto terapii reagují [108]. V tuto chvíli se zdá, že u vybraných diagnóz je pozitivní odpověď na terapii okolo 25 % pacientů, zatímco u zbylých pacientů je klinický přínos minimální [109]. Snahou většiny současných studií je tedy modulace nádorového mikroprostředí tak, aby bylo zvýšeno procento pacientů, kteří na léčbu odpovědí [110]. Druhým úskalím je výskyt autoimunitně podmíněných nežádoucích reakcí, které se ukázaly být novinkou ve světě onkologie a jejichž management spočívá především v nutnosti imunosupresivní terapie [111]. Přesto jsou checkpoint inhibitory významnou součástí moderní léčby nádorových onemocnění a jejich začlenění do léčebných algoritmů je žádoucí.

Další imunoterapeutické přístupy jsou například adoptivní T-buněčný transfer či terapie prostřednictvím CAR T-buněk [104, 112]. V obou případech jsou opět terčem léčby T-buňky. V případě adoptivního transferu jsou T-buňky izolovány z periferní krve nebo z nádoru pacienta, v laboratoři následně množeny do obrovských počtů, purifikovány, za pomoci dendritických buněk stimulovány nádorovými antigeny a následně injikovány zpět pacientovi [104]. Adoptivní transfer je vysoce personalizovanou léčbou, která je nyní předmětem klinických studií. Pro klinickou praxi byly již schváleny takzvané CAR T-buňky, a to především pro léčbu hematologických malignit. CAR T-buňky jsou geneticky modifikované T-buňky, na jejichž TCR receptor je navázaná monoklonální protilátka, aby k vazbě a aktivaci T-buňky docházelo bez nutnosti MHC molekul, které jsou jinak pro aktivaci T-buňky nezbytné [112]. CAR T-buňky představují průlom v léčbě akutní leukémie, a i přes jejich vysokou efektivitu je jejich široké užívání limitováno cenou přípravku [112, 113].

Pro solidní tumory se tedy zdá být metodou volby terapie checkpoint inhibitory ať už v monoterapii, či v kombinované léčbě [114]. Data naznačují, že checkpoint inhibitory v kombinacích vykazují vyšší účinnost než v monoterapii, a dokonce některé systémové terapie, jako jsou například chemoterapie či radioterapie, zvyšují citlivost nádorů k účinkům imunoterapie [115].

## 2  HYPOTÉZA A CÍLE

Cílem této práce bylo poskytnout detailní pohled na nádorové mikroprostředí různých podtypů sarkomů měkkých tkání a definovat faktory, které jsou asociované s klinicko-patologickými charakteristikami konkrétních malignit, za účelem rozšíření znalostí o tomto vzácném nádorovém onemocnění.

Naše primární hypotéza předpokládala, že imunitní infiltrace měkkotkáňových sarkomů závisí na histologickém podtypu nádoru a že větší množství klinických parametrů, jako jsou například lokalita nádoru nebo chirurgické komplikace, má vliv na riziko lokální recidivy a generalizace. Vytvořili jsme přehled všech dostupných informací o nádorovém mikroprostředí měkkotkáňových sarkomů, detailně jsme zkoumali histologickou entitu zvanou solitární fibrózní tumor a v neposlední řadě jsme vytvořili in vitro podmínky pro testování různých typů imunoterapie u vybraných pacientů s měkkotkáňovým sarkomem.

## 3   METODIKA

Popis konkrétních metodik je součástí publikovaných prací.

**Metody strukturovaně:**

### a) Práce s klinicko-patologickými daty

Pro účely hodnocení biologického chování nádoru, jeho potenciálu k zakládání metastáz a rizika lokálních recidiv jsme získávali z centrální databáze Fakultní nemocnice Motol UNIS data o histologickém typu nádoru a jeho platný grade a stage. Z pacientských dat jsme dále retrospektivně dohledali v našich pečlivě vedených kohortách záznamy týkající se operačních komplikací, počtu chybných diagnóz, předchozí dispenzarizace na jiných pracovištích, ale i stěžejní klinické proměnné, jako jsou věk či pohlaví pacienta. Soubory klinických dat byly anonymizovány a všechny identifikovatelné odkazy na jednotlivce byly odstraněny. Tento výzkum probíhal se souhlasem Etické komise.

### b) Izolace tumor-infiltrujících lymfocytů (TILs)

Tumor-infiltrující lymfocyty byly izolovány bezprostředně po operaci z nativních tumorů po jejich vyjmutí z těla. Tato izolace probíhala za pomoci mechanického rozrušení tkáně nůžkami a následné kultivaci tkáňových fragmentů za přítomnosti enzymů degradujících extracelulární matrix. Tento postup umožnil rozrušení tkáně a následné pasírování tkáňové suspenze přes sto mikrometrů buněčné síto umožnilo získání buněčné suspenze s tumor-infiltrujícími lymfocyty.

## c) Kultivace TILs a měření jejich aktivity

U vybraných pacientů jsme získané TILs kultivovali in vitro za přítomnosti terapeutických monoklonálních protilátek. Doba působení terapeutik činila zpravidla 24 hodin. Cytotoxické schopnosti buněk byly testovány nepřímo prostřednictvím produkce cytotoxických cytokinů v buněčných suspenzích.

# 4 VÝSLEDKY A DISKUZE

V této kapitole budou představeny výsledky výzkumu formou komentářů k publikovaným článkům. Jednotlivé výstupy této práce byly publikovány v odborných impaktovaných časopisech s recenzním řízením a zásadně rozšířily znalosti o problematice diagnostiky, terapie a analýzy nádorového mikroprostředí měkkotkáňových sarkomů. Výsledky jsou diskutovány v kontextu předpokládaných cílů a dosavadních informací k problematice.

Plné znění vědeckých prací je přiloženo za každým komentářem.

## 4.1 „Diagnostic challenges and treatment options in patients with solitary fibrous tumor: A single-center observational study"

OZANIAK A, HLADIK P, LISCHKE R, STRIZOVA Z.

Solitární fibrózní tumor (SFT), v minulosti také označován jako hemangiopericytom, je vzácný mezenchymální nádor s incidencí jenom kolem 2 případů na 1 000 000 lidí ročně. Některé podtypy SFT se vyznačují maligním metastatickým potenciálem. SFT patří k pomalu rostoucím nádorům, které se mohou vyskytovat kdekoliv na těle. Klinicky se prezentují především symptomy z působení tlaku primárního nádoru nebo metastázy v sousedních tkáních. Nejčastěji se vyskytují v oblasti hrudníku.

V naší studii jsme sdíleli naše klinicko-patologické zkušenosti s osmnácti pacienty s touto vzácnou diagnózou. Studie zahrnovala pacienty operované v letech 2014–2021. Medián sledování byl 36 měsíců. U 17 % našich pacientů se předoperační diagnóza neshodovala s pooperační histologií. Četnost chybných diagnóz byla proto značně vysoká, avšak korelovala s dříve publikovanými studiemi. Pozorovali jsme vyšší riziko lokální recidivy u pacientů, kteří na našem oddělení podstoupili operaci pro recidivující SFT, než u pacientů, kteří podstoupili operaci primární SFT (2/10 versus 4/6). Pooperační komplikace byly spojeny s mimohrudní lokalizací SFT. Pacienti s končetinovou lokalizací solitárního fibrózního tumoru měli obecně nejlepší prognózu a žádné známky recidivy či metastáz ve sledovaném období. Tato zjištění však byla omezena počtem účastníků studie a jejich relevance bude vyžadovat ověření dalším výzkumem. Pacienti, kteří vstupovali do studie již s recidivou solitárního fibrózního

tumoru, vykazovali signifikantně vyšší šanci k další recidivě (67 %) a k systémové progresi došlo u 33 % pacientů, kteří byli přijati a léčeni pro recidivující SFT.

Základem léčby u lokalizovaného SFT zůstává radikální operace, kde je získání negativních resekčních okrajů nejdůležitějším faktorem prevence recidivy onemocnění. Dosažení radikální resekce s mikroskopicky negativním okrajem může být svízelná v důsledku přítomnosti kritických anatomických struktur.

Samotná radioterapie může významně zlepšit celkové přežití pacientů a kombinace chirurgického výkonu a radioterapie signifikantně snižuje riziko lokální recidivy. Domníváme se, že součástí sofistikovaného terapeutického schématu u SFT by měla být multimodální terapie, jejíž součástí by měly být také cílené terapie a imunoterapie. V současné době je třeba navrhnout nové klinické studie zaměřené na léčbu této vzácné choroby.

# Diagnostic challenges and treatment options in patients with solitary fibrous tumor: A single-center observational study

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Introduction: Solitary fibrous tumor (SFT) is an extremely rare disease with a high misdiagnosis rate and a potentially malignant biologic nature. We have collected and analyzed data from 18 SFT patients to provide a deeper insight into this uncommon disease entity.

Methods: In our study, 18 patients who had undergone surgery between April 2014 and December 2021 for the diagnosis of SFT were evaluated. The collected data for each patient included the location of the SFT, the preoperative diagnosis, the definitive histological diagnosis, the presence of postoperative complications, the time of recurrence, the time of systemic progression, the type of treatment, and the survival rate. The median follow-up was 36 months.

Results: In three patients, the preoperative diagnosis did not correlate with the definitive histology of SFT. In patients with the limb location of SFT, no signs of recurrence nor distant metastases were seen within the study period. In total, 50% of the postsurgical complications were associated with the abdominal location of the SFT. In newly diagnosed SFT patients, two patients (20%) developed local recurrence, and the median time until recurrence was 22.5 months. Out of patients that were admitted and operated on for recurrent SFT, 67% relapsed, and the median time to relapse was 9.5 months. The systemic progression of the disease was observed in 33% of patients treated for recurrent SFT.

Conclusion: In our study, the misdiagnosis rate was high and correlated with previously published studies. Postsurgical complications were associated with the extrathoracic location of SFT. The mainstay of SFT treatment remains radical surgery, although radiotherapy alone can significantly improve overall survival. Clinical trials are urgently needed to evaluate the potential effect of other treatment modalities, such as immunotherapy and targeted therapy, in SFT patients.

## Introduction

Solitary fibrous tumors (SFTs) are rare fibroblastic mesenchymal neoplasms that arise in various anatomic locations (1). Due to similarities to other soft tissue tumors, SFTs can often be difficult to diagnose and treat (2). The biological behavior of SFTs is uncertain; nevertheless, metastatic potential has already been observed (1). In previous studies, most SFTs were shown to be associated with an indolent clinical course but also displayed patterns of distant metastases in up to 40% of patients at 10 years of follow-up (3–5). Moreover, relapse-free survival in a follow-up period of approximately 20 years was reported to be less than 20% (5). Several risk classification models were created and eventually proven as accurate in predicting the risk of disease recurrence (6–8). The established risk factors mostly included disease-specific features, such as tumor size and location, mitotic count, and patient's individual characteristics, such as age and sex (6–8).

In most cases, the size of SFTs ranges from 7 to 10 cm (1). Size and location are presumably by far the most important prognostic factors (4). In particular, size greater than 8 cm was associated with both local and distant recurrences. Depending on the size, the disease may exhibit nonspecific symptoms due to the compression of surrounding organs. However, most SFTs are painless and slow-growing (9). Extremely rare are paraneoplastic syndromes, such as Doege-Potter syndrome or Pierre-Marie-Bamberger syndrome (10). SFTs can be divided according to several criteria (1, 2). Conventional classification divides SFTs according to their location into intrathoracic (pleuropulmonary) SFTs, accounting for over 30% of the cases, intra-abdominal SFTs, SFTs of the head and neck (intracranial or extracranial) and SFTs of the soft tissues (1, 2). The importance of disease location in the patient's prognosis was pronounced by multiple studies; however, the aggressive behavior of SFTs has been mostly associated with two SFTs locations, intrathoracic and retroperitoneal/intra-abdominal locations (4, 6, 11).

SFTs can also be alternatively classified according to their histological features (1, 2, 12). Interestingly, it has been shown that SFTs that lack malignant histological features in primary resection specimens may still acquire these features at the time of recurrence (13). The diagnosis of SFT is usually made upon a combination of imaging techniques, pretreatment biopsy, and histopathological evaluation (1). Here, we present our single-center experience in the treatment and management of this rare disease in 18 patients.

## Materials and methods

To analyze the clinicopathological features of SFTs, all patients who had undergone surgery between April 2014 and December 2021 for the diagnosis of SFT were evaluated. A total of 18 patients were enrolled in the study and provided written informed consent. The exclusion criteria for participation in the study were the presence of comorbid malignant conditions, unclear primary diagnosis, refusal to give informed consent, age <18, and pregnancy at the time of study initiation. However, none of the study participants met the exclusion criteria. Of the 18 study participants, 10 patients had newly diagnosed SFT without any previous treatment, 6 patients were admitted to our hospital for local recurrence of SFT, and 2 patients were admitted for a single metastasis of SFT. In recurrent/metastatic SFT, data regarding the previous SFT operation, including radicality of resection and perioperative/postoperative complications, were not evaluated. The patients' clinicopathological data, including sex, age, preoperative and postoperative histology, and the provided therapy, were collected and analyzed. In patients who were surgically treated, the status of the resection margins was documented. The median age of our patients was 55 years, ranging from 33 to 80 years. The female:male ratio was 10:8. The collected data for each patient included the location of the SFT, preoperative diagnosis, definitive histological diagnosis, presence of postoperative complications, time of recurrence, time of systemic progression, systemic treatment given, and the survival rate. All patients' data are summarized in Table 1. Statistical analysis was performed by GraphPad Prism 6 (GraphPad, La Jolla, CA) and Microsoft Excel (Microsoft for Windows, 2013). $P < .05$ was considered significant. For graphical presentation, Microsoft Excel and BioRender software were used.

## Results

### Preoperative misdiagnosis rate is high

Owing to the complexity of histological features exhibited by SFTs, we aimed to evaluate the misdiagnosis rate in our study cohort. In three (16.67%) of our patients, the preoperative diagnosis did not correlate with the definitive histology of SFT. These three patients were primarily diagnosed with synovial sarcoma, pleural tumor, and peripheral nerve-sheath tumor (PNST). Thus, our data indicate that the preoperative diagnosis of SFT may cause difficulties, and therefore, the initial step in the differential diagnosis should contain the exclusion of other disease entities, such as sarcoma, gastrointestinal stromal tumor (GIST), and other diseases, as shown in Figure 1. Since biopsy highly contributes to the diagnostic process, in 12 (66.67%) of our SFT patients, a preoperative biopsy was performed. Six (33.33%) patients did not undergo preoperative biopsy, mainly due to the medical history of previous SFT at the same location.

TABLE 1 Data associated with the provided surgery and the outcome of the patients.

| Clinical data of the patients | |
| --- | --- |
| Age, median (range) | 55 (33–80) |
| Sex | |
| Female | 10 (55.56%) |
| Male | 8 (44.44%) |
| Primary site | |
| Intrathoracic | 14 (77.78%) |
| Intra-abdominal | 2 (11.11%) |
| SFT of soft tissues | 2 (11.11%) |
| Operation | |
| Primary SFT | 10 (55.56%) |
| Locally recurrent SFT | 6 (33.33%) |
| Systemic SFT | 2 (11.11%) |
| Radicality | |
| R0 | 17 (94.44%) |
| R1 | 1 (5.56%) |
| R2 | 0 (0%) |
| Complications | |
| Bleeding | 3 (16.67) |
| Infection | 1 (5.56%) |
| Without complications | 14 (77.78%) |
| Local recurrence | |
| Primary SFT | 2 (20%) |
| Recurrent SFT | 4 (66.67%) |
| Systemic SFT | 0 (0%) |
| Systemic progression | |
| Primary SFT | 1 (10%) |
| Recurrent SFT | 2 (33.33%) |
| Systemic SFT | 0 (0%) |
| Follow-up, median (range) | 36 (5–72) |

SFT, solitary fibrous tumor.

## Intrathoracic SFTs are more frequent than extrathoracic SFTs

The surgical management of SFT depends on the anatomic location of the tumor (12, 14, 15). Several locations of SFTs have been reported, including the salivary gland, larynx, orbits, liver, and pancreas (15). In our study of 18 SFT patients with a median age of 55 years, 14 (77.78%) patients had an intrathoracic location of the tumor, 2 patients (11.11%) had an abdominal location, and 2 (11.11%) patients had an SFT located in the lower extremities (Figure 2, upper left and upper right section, created with BioRender.com, No. JV244CL242). In patients with limb location of SFT, neither signs of recurrence nor distant metastases were observed within the study period. The same clinical course was observed in one of the patients with an abdominal location of

SFT. The second patient with an abdominal location of SFT died of an unknown cause 5 months after the surgical treatment; however, there were neither signs of SFT recurrence nor systemic progression. In our study, the incidence of SFTs in the intrathoracic area was higher than that of those in other locations, which was in accordance with the study by Zhanlong et al. evaluating diverse SFT locations in 20 patients (16).

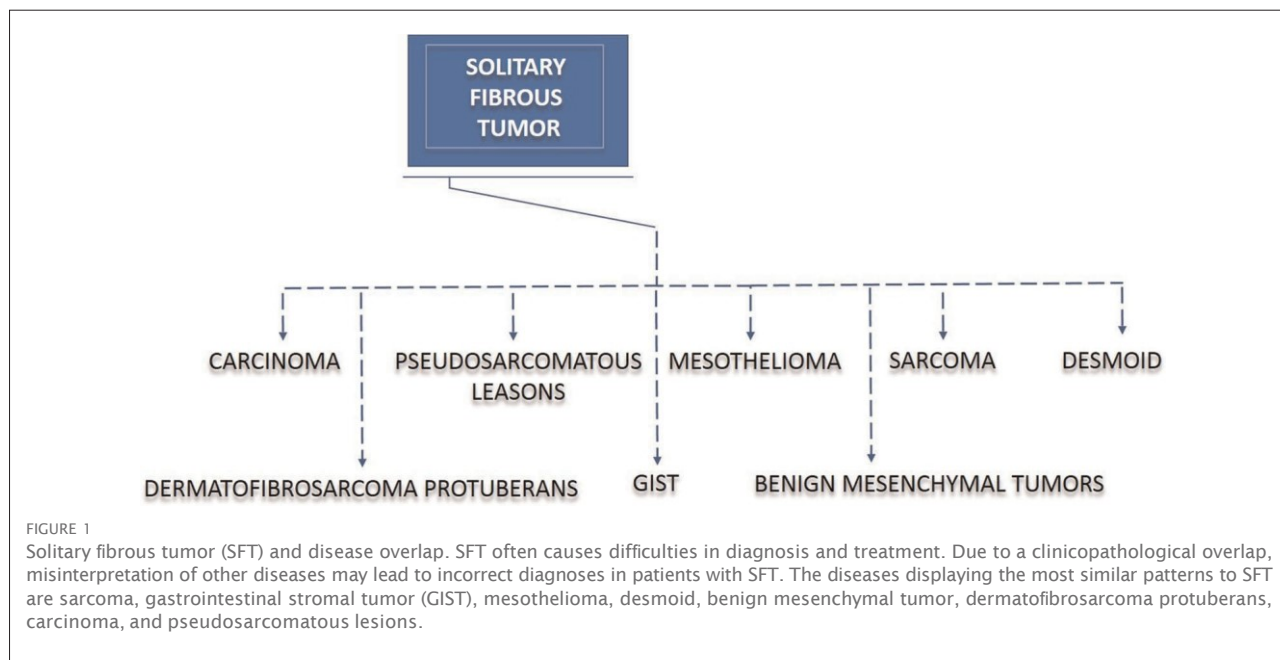## Complete surgical excision leads to long-term survival

In SFT, surgery is the leading treatment option for localized disease (12, 15). The surgical management is similar to that of soft tissue sarcomas, and thus, obtaining negative resection margins is crucial (1, 12, 15).

In all 18 patients enrolled in our study, surgical resection was provided. Each surgery was performed by a specialist in oncologic surgery and assisted by a specialized thoracic/abdominal surgeon. Negative resection margins were achieved in 17 (94.44%) out of 18 patients. Although it has already been shown that a complete surgical excision leads to long-term survival, in one of our patients, the complete surgical resection was not accomplished (1, 12, 15). In this patient, the main tumor mass required a 10-cm resection of the sixth and seventh rib. However, with the further progress of the operation, multiple foci on the parietal, mediastinal, and diaphragmatic pleura were observed and led to a termination of the operation after a multidisciplinary assessment. In this patient, only the largest tumor mass, which was firmly fixed to the chest wall, was resected.

Even though local recurrence and seeding of the tumor on the peritoneal or pleural surface have been reported to have a significant association with the positive resection margins, in our study, the patient with positive resection margins did neither exhibit signs of recurrence nor distant metastases within the study period (26 months follow-up) (14). This was in contrast with the data of previously published studies (1, 12, 14).

## Surgical complications were mostly associated with the extrathoracic location of the SFT

Our previous observations prompted us further to investigate the surgical complications in our study cohort. A postsurgical complication occurred in 4 patients (22.22%) out of 18 study participants. Three complications were of hemorrhagic origin. One was an infectious complication. Two (50%) of the four documented complications were associated with the abdominal location of the SFT. The extrathoracic location of the SFT was shown by multivariate analyses to be

FIGURE 1
Solitary fibrous tumor (SFT) and disease overlap. SFT often causes difficulties in diagnosis and treatment. Due to a clinicopathological overlap, misinterpretation of other diseases may lead to incorrect diagnoses in patients with SFT. The diseases displaying the most similar patterns to SFT are sarcoma, gastrointestinal stromal tumor (GIST), mesothelioma, desmoid, benign mesenchymal tumor, dermatofibrosarcoma protuberans, carcinoma, and pseudosarcomatous lesions.

an independent indicator of increased risk of disease recurrence (14). However, in our study, neither a tendency toward increased mortality nor toward disease recurrence was observed in abdominal/limb SFTs.

The incidence of surgical complications in each patient together with the clinicopathological data of the study participants is graphically presented as a heatmap (Figure 3).

## Patients treated for recurrent SFT tended to relapse more often and earlier than patients with newly diagnosed SFT

In our cohort, we evaluated patients with local recurrence and patients with systemic progression to investigate the risk factors associated with these findings. Overall, the study participants with primary SFTs that were treated at our department had a low rate of systemic progression after surgical treatment. Out of 10 patients who underwent surgery for primary SFT, 2 (20%) patients developed a local recurrence and 1 (10%) patient presented with a systemic progression of the disease, as shown in Figure 2, lower right section. The systemic progression occurred 13 months after the surgery. Out of 6 patients who were admitted and operated on at our department for local recurrence of SFT, 4 (66.67%) patients presented with another episode of local SFT recurrence after being surgically treated, and 2 (33.33%) patients developed distant metastases (Figure 2, lower right section). The presence of metastases was associated with previous local recurrence. Two out of 18

patients who were admitted and operated on for metastatic disease did neither develop local recurrence nor distant metastases and remained disease-free in our study at 12 and 22 months. In patients who developed systemic progression within the study period, metastases of SFT were observed in the thorax ($n = 2$) or in both the thorax and abdomen ($n = 1$). The age of the patients did not correlate with the risk of local recurrence nor with the systemic progression. As shown in Figure 4 (created with BioRender.com, No. JW23XVB7UY), patients who were admitted and operated on for recurrent SFT (6 out of 18) tended to relapse more often (66.67%) and earlier (median 9.5 months, ranging from 5 to 19 months) than patients operated on for newly diagnosed SFT, where only 20% of the patients had local recurrence and the median time until disease recurrence was 22.5 months. Systemic progression of the disease was observed in 33.33% of patients who underwent surgery for recurrent SFT and in 10% of patients with newly diagnosed SFTs, as shown in Figure 2, lower left section. However, we admit that our study cohort is rather small due to the rarity of the disease entity, and therefore, further investigation is needed.

## Radiotherapy, targeted therapy, and immunotherapy reveal a great potential in the treatment of SFT

Chemotherapy can be given in both neoadjuvant and adjuvant settings; however, it has only limited efficacy in
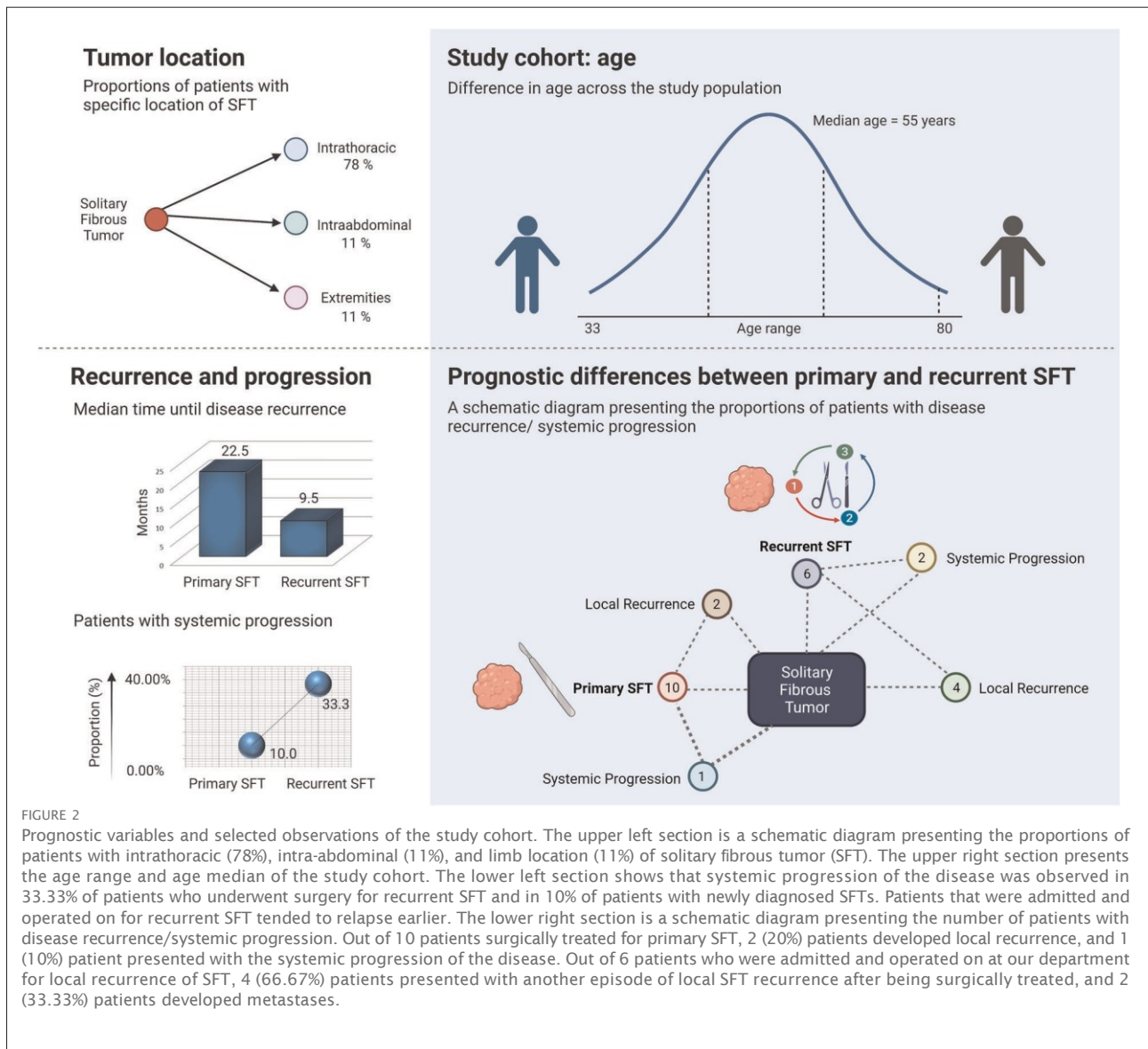
FIGURE 2
Prognostic variables and selected observations of the study cohort. The upper left section is a schematic diagram presenting the proportions of patients with intrathoracic (78%), intra-abdominal (11%), and limb location (11%) of solitary fibrous tumor (SFT). The upper right section presents the age range and age median of the study cohort. The lower left section shows that systemic progression of the disease was observed in 33.33% of patients who underwent surgery for recurrent SFT and in 10% of patients with newly diagnosed SFTs. Patients that were admitted and operated on for recurrent SFT tended to relapse earlier. The lower right section is a schematic diagram presenting the number of patients with disease recurrence/systemic progression. Out of 10 patients surgically treated for primary SFT, 2 (20%) patients developed local recurrence, and 1 (10%) patient presented with the systemic progression of the disease. Out of 6 patients who were admitted and operated on at our department for local recurrence of SFT, 4 (66.67%) patients presented with another episode of local SFT recurrence after being surgically treated, and 2 (33.33%) patients developed metastases.

the treatment of SFT (1, 17). The superior efficacy of one approach over another (neoadjuvant/adjuvant) has not been reported. None of our patients received neoadjuvant chemotherapy. One of our patients received chemotherapy in an adjuvant setting after the failure of other therapeutic modalities. This patient died one year after the initial diagnosis.

Unfavorable tumor behavior serves as an indication of radiotherapy administration. Two of our patients received adjuvant radiotherapy after a local recurrence of the disease. One of the patients is still disease-free at 26 months after the initial diagnosis. The second patient died 39 months after the initiation of radiotherapy. None of our patients received targeted therapy or immunotherapy.

# Discussion

SFTs are rare mesenchymal tumors with a risk of local recurrence and a metastatic potential (1, 2). Although SFTs have been reported at almost every anatomic site, the intrathoracic location is the most prevalent one (18). SFTs belong to slow-growing tumors but may eventually cause pressure on the adjacent tissues (19). In our study, we shared our experience with 18 SFT patients.

Our data indicated that the preoperative misdiagnosis rate was high and corroborated that of previously published studies by Chu et al. and Kim et al. (20, 21).

In 17% of our patients, the preoperative diagnosis did not match the postoperative histology. Thus, our findings were in accordance with a previously published study on high
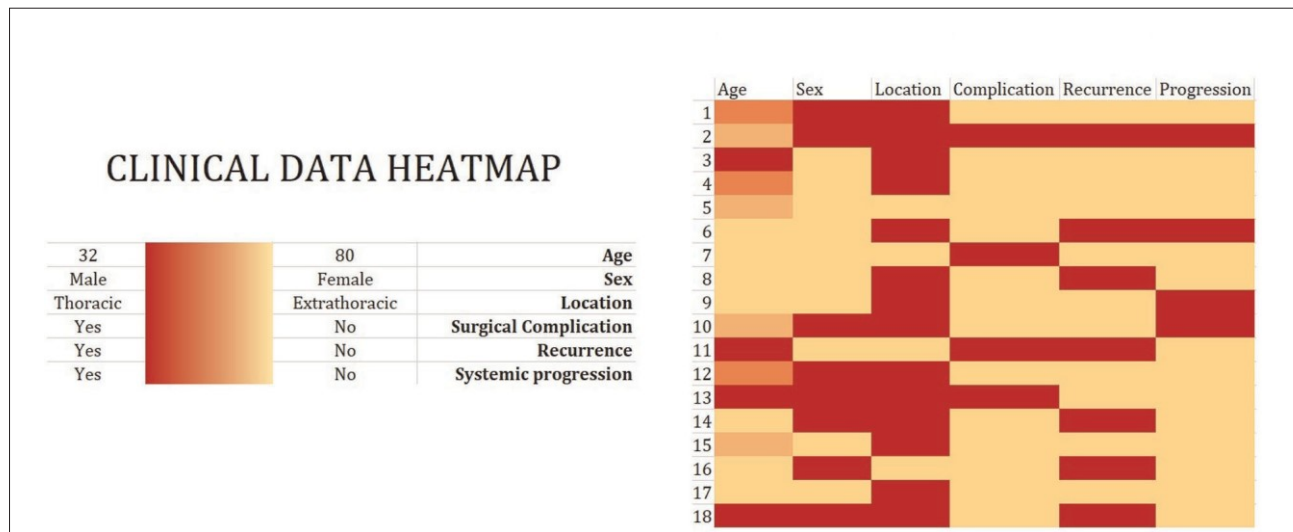
FIGURE 3
Clinical data heatmap. Eighteen patients underwent surgical treatment between April 2014 and December 2021 for the diagnosis of solitary fibrous tumor (SFT). Clinical data, including age, sex, tumor location, and disease recurrence/systemic progression, are visualized in a heatmap. The color intensity shows the magnitude of a selected phenomenon.
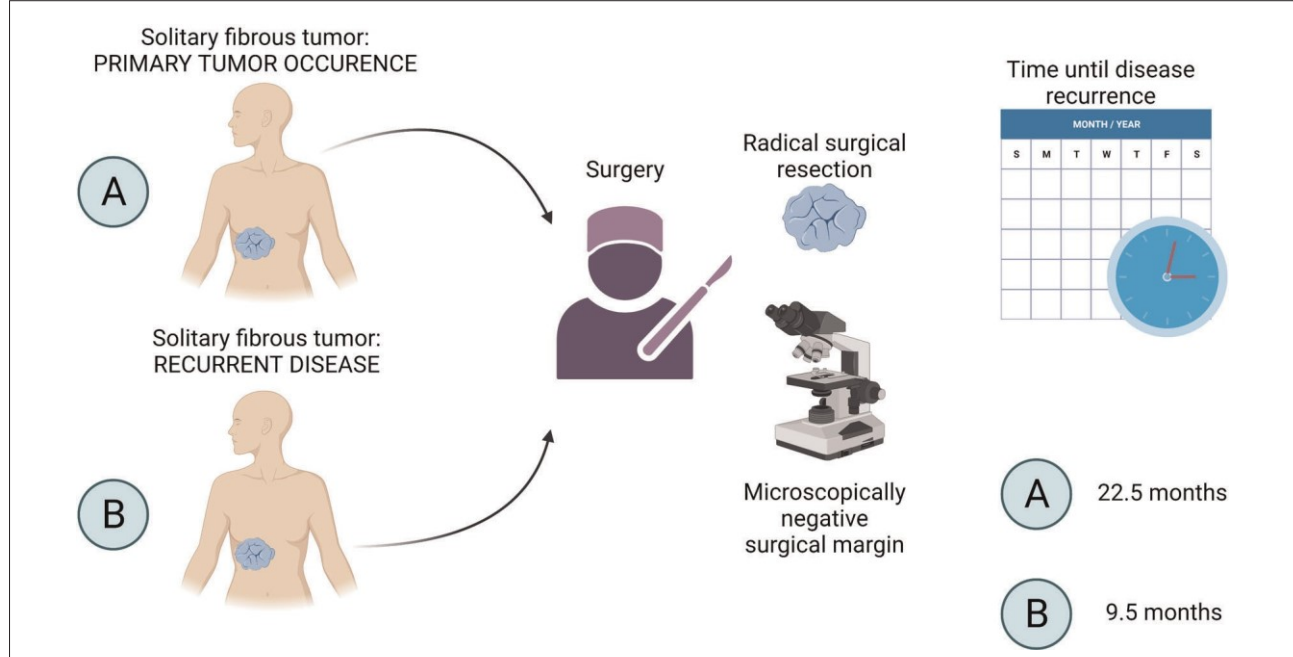


FIGURE 4
Time until disease recurrence. Patients with recurrent SFT (B) tended to relapse earlier (median 9.5 months, ranging from 5 to 19 months) than patients operated on for newly diagnosed SFT (A). The median time until disease recurrence in (A) patients was 22.5 months. SFT, solitary fibrous tumor.

misdiagnosis rates in SFTs (20). A compelling point of discussion was raised in a study by Hohenforst-Schmidt et al., revealing the importance of appropriate radiological examination as a part of the complex differential diagnosis (22). This, however, still presents a great hurdle since the imaging findings of SFTs are similar to those of other blood-rich tumors. Hence, to date, the risk of SFT misdiagnosis remains significant (22–24).

Biopsy highly contributes to the diagnostic process (1, 25, 26). In our study, the biopsy was not performed in patients

with a previous medical history of SFT. This study group was directly operated on and included only those with the intrathoracic location of SFT. Saynak et al. suggested that a video-assisted thoracoscopic (VATS) biopsy may be considered an optimal approach to obtain a precise preoperative diagnosis (24). However, VAST biopsy is mostly superficial and thus, may not be sufficient in the diagnosis of SFT (27). We believe that VATS biopsy is particularly beneficial for the validation of the possible resectability of the tumor.

For histological verification, the standard diagnostic approach should include multiple core needle biopsies, possibly by using ≥14–16 G needles. Biopsy in deep-sealed tumors should be CT navigated and in superficial tumors, performed by a Tru-Cut needle. For superficial tumors ≤3 cm, excisional biopsy is the most convenient option (26, 28, 29).

In our SFT patients, we attempted to provide a complete surgical resection where possible. Negative resection margins were achieved in 94.44% of the patients. The need for obtaining negative resection margins stems from the fact that SFTs have uncertain biological behavior and a high rate of local recurrences (1, 4, 10).

Nonetheless, the surgical treatment may be difficult due to the abundant blood supplies that are often seen in SFTs (30). Wang et al. reported a case of SFT with vessel abnormalities in the tumor tissue, such as arteriovenous short circuits, which contributed to portal vein disease (17). Moreover, these abnormalities were neither obvious in the blood examination, electrocardiogram examination, nor in the chest radiograph examination before the laparotomy (17).

We observed a higher risk of local recurrence in patients who underwent surgery at our department for recurrent SFT than in patients who underwent surgery for primary SFT. In our study, the postoperative complications were associated with the extrathoracic location of the tumor. These findings, however, were limited by the number of study participants and required verification by further research. In addition, previous studies have demonstrated a tendency toward increased mortality in abdominal SFTs as compared to the SFTs in the limbs (14). This has not been observed in our study.

The late presentation of abdominal SFTs was discussed as a factor contributing to a higher mortality (14). We, however, also comment on the fact that providing a wide surgical excision and obtaining negative surgical margins is far more challenging in the abdomen than in the limbs.

Radiotherapy can be given as both neoadjuvant and adjuvant treatment (1). However, both adjuvant radiotherapy and chemotherapy are not routinely required in SFTs. In our experience, the incorporation of radiotherapy in SFT treatment should be considered in each patient who has undergone surgical excision without achieving negative surgical margins. In our study, two patients were given adjuvant radiotherapy, with only one of these patients being alive at the study termination. We highly support the study by Haas et al. on the efficacy of radiotherapy in sarcoma patients and patients with sarcomatous lesions (31). Moreover, studies have reported a promising efficacy of systemic therapies, such as bevacizumab, a humanized recombinant antibody against vascular endothelial growth factor (VEGF), together with temozolomide, an alkylating chemotherapeutic in SFT patients (32). Both pazopanib, an anti-VEGF receptor agent, and sunitinib, a tyrosine kinase inhibitor, have also shown potential in SFT treatment (33, 34).

Immunotherapy has not been approved for SFT so far. However, a single case report of a patient treated with an anti-PD-1 checkpoint inhibitor has shown remarkable results (35). The efficacy of different immunotherapies, thus, remains to be clarified.

## Conclusion

SFTs are diagnostically challenging malignancies with a high rate of misdiagnoses. Establishing the correct diagnosis requires a complex integration of clinical and histopathological features of the tumor, together with ruling out more common disease entities. Only a wide differential diagnosis excluding other potentially malignant tumors, such as soft tissue sarcomas, carcinomas, and/or GISTs, leads to accurate treatment selection.

The mainstay of SFT treatment remains radical surgery, where obtaining negative resection margins is the most important factor preventing the disease recurrence.

While radiotherapy alone can significantly improve the overall survival of patients, we believe that more therapies, mainly targeted therapy and immunotherapy, should become a part of the sophisticated therapeutic scheme in SFT (14, 19). Currently, a global consensus on the treatment SFTs is lacking and randomized clinical trials need to be designed for these rare disease entities. A multidisciplinary team approach should prevent the false management of these tumors.

## Data availability statement

The authors are happy to share the raw data supporting the conclusions of this article on request to the corresponding author.

## Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee for Multi-Centric Clinical

Trials of the University Hospital Motol (Reference No. EK-948/20). The patients/participants provided their written informed consent to participate in this study.

## Author contributions

## Funding

## Acknowledgment

## Conflict of interest

## Publisher's note

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## 4.2  „Novel Insights into the Immunotherapy of Soft Tissue Sarcomas: Do We Need a Change of Perspective?"

OZANIAK A, VACHTENHEIM J JR, LISCHKE R, BARTUNKOVA J, STRIZOVA Z.

Měkkotkáňové sarkomy jsou extrémně biologicky a klinicky různorodé nádory s více než 80 histologickými podtypy. Vzhledem k extrémní vzácnosti těchto nádorů klinické studie často hodnotí všechny typy STS společně, což může přispět k nejednoznačným a někdy až kontroverzním výsledkům při evaluaci účinnosti terapií. Léčba metastatických STS se v posledních letech zásadně nezměnila a se současnými konvenčními terapiemi jsou šance na zlepšení celkového přežití velmi malé.

Nádorové mikroprostředí STS je infiltrováno různými podíly imunitních buněk, které umožňují stratifikovat pacienta podle imunitních parametrů. CD8+ T-buňky patří k hlavním protinádorovým hráčům v nádorovém mikroprostředí a vysoký podíl CD8+ T-buněk se stejně jako exprese checkpoint molekul staly nezbytnými předpoklady pro účinnost imunoterapie checkpoint inhibitory.

Vytvořili jsme přehledový článek, ve kterém jsme nejprve detailně popsali nejdůležitější imunitní buňky nacházející se v nádorovém mikroprostředí sarkomů měkkých tkání a následně jsme prezentovali klinické studie zaměřené na konkrétní buněčnou populaci u STS. Pleomorfní sarkom a myxofibrosarkom mají jednu z nejvyšších infiltrací CD8+ T-buňkami a nejvyšší expresi PD-1. Z tohoto důvodu se předpokládá dobrá účinnost checkpoint inhibitorů u těchto histologických podtypů. Pleomorfní sarkom má dokonce imunitní repertoár srovnatelný s maligním

melanomem, což naznačuje jeho vhodnost pro aplikaci imunoterapeutik. Leiomyosarkomy a liposarkomy mají napříč studiemi obecně nízkou infiltraci CD8$^+$ T-buňkami, což se odráží ve velmi neuspokojivých výsledcích klinických studií. Kombinované terapie checkpoint inhibitory, jako jsou například nivolumab plus pembrolizumab, ukázaly zvýšení klinické odezvy u pacientů s STS. Mezi další slibné terapie v klinických studiích patří adoptivní buněčný transfer nebo CAR T-buněčná terapie.

Leiomyosarkomy mají relativně vysokou zátěž T-regulačními buňkami a tato infiltrace pozitivně koreluje se stadiem, gradem a hloubkou nádoru. Navíc u nediferencovaných pleomorfních sarkomů se infiltrace T-regulačními buňkami zvyšuje po neoadjuvantní radioterapii a ovlivňuje celkové přežití pacientů. Zatímco intratumorální NK buňky vykazují četnou aktivaci, ale i znaky vyčerpání v nádorovém mikroprostředí, NK buňky v periferní krvi pacientů s STS byly popisovány jako funkčně poškozené.

Makrofágy dominují nádorovému mikroprostředí STS a převyšují počty ostatních imunitních buněk.

Je třeba poznamenat, že změny v zastoupení intratumorálních makrofágů byly prokázány u pacientů po neoadjuvantní chemoterapii. Terapie zaměřené na makrofágy jsou tedy u STS velmi slibné.

Se současnými znalostmi různých populací imunitních buněk infiltrujících STS nádory by se probíhající klinická praxe mohla zásadně změnit a umožnit stratifikaci pacientů na základě imunitního repertoáru nádorového mikroprostředí. Jak ukazují klinické studie, imunoterapie lze snadno kombinovat a chemoterapie může sloužit jako silný nástroj ke zvýšení citlivosti nádorového mikroprostředí k účinkům imunoterapie.

*Review*

# Novel Insights into the Immunotherapy of Soft Tissue Sarcomas: Do We Need a Change of Perspective?

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**Abstract:** Soft tissue sarcomas (STSs) are rare mesenchymal tumors. With more than 80 histological subtypes of STSs, data regarding novel biomarkers of strong prognostic and therapeutic value are very limited. To date, the most important prognostic factor is the tumor grade, and approximately 50% of patients that are diagnosed with high-grade STSs die of metastatic disease within five years. Systemic chemotherapy represents the mainstay of metastatic STSs treatment for decades but induces response in only 15–35% of the patients, irrespective of the histological subtype. In the era of immunotherapy, deciphering the immune cell signatures within the STSs tumors may discriminate immunotherapy responders from non-responders and different immunotherapeutic approaches could be combined based on the predominant cell subpopulations infiltrating the STS tumors. Furthermore, understanding the immune diversity of the STS tumor microenvironment (TME) in different histological subtypes may provide a rationale for stratifying patients according to the TME immune parameters. In this review, we introduce the most important immune cell types infiltrating the STSs tumors and discuss different immunotherapies, as well as promising clinical trials, that would target these immune cells to enhance the antitumor immune responses and improve the prognosis of metastatic STSs patients.

**Keywords:** sarcoma; immunotherapy; checkpoint inhibitors; adoptive transfer; trabectedin; IL-15; tumor microenvironment; TILs; immune cells

## 1. Introduction

Soft tissue sarcomas (STSs) are a heterogeneous group of rare tumors arising from mesenchymal tissues [1]. STSs can originate from any human body location and, with more than 80 histological subtypes of STSs, data regarding novel biomarkers of strong prognostic and therapeutic value are very limited [2,3]. The most prevalent histological subtypes of STSs include liposarcoma, undifferentiated pleomorphic sarcoma, and leiomyosarcoma [4]. Most STS occur spontaneously and present as an asymptomatic soft tissue mass [5,6]. Nevertheless, certain factors, such as exposure to radiation and chemicals or genetic aberrations, were also previously associated with the risk of developing STSs [6].

The American Joint Committee on Cancer (AJCC) staging system for STSs relies on the histologic grade, the tumor size and depth, and the presence of distant or nodal metastases [7–9]. To date, the most important prognostic factor is the tumor grade [9]. Approximately 50% of patients that are diagnosed with high-grade STSs die of metastatic disease [10]. Most STSs are known for early hematogenous metastasizing [11]. The disease rarely affects the lymphatic system, but the impairment of the lymph nodes is a sign of high tumor aggressiveness [12,13]. The predominant site of metastases are the lungs. However, retroperitoneal STSs also tend to metastasize to the liver [14,15]. Other metastatic sites commonly include the bones and the brain [16,17].

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Patients with STSs are managed according to the generally accepted guidelines and those with localized and resectable diseases are treated by surgery [18]. The mainstay of STS treatment is a complete surgical resection of the tumor with ensured negative margins [19]. Although major improvements in the local control rates are achieved, the success of surgery critically depends on the tumor location, tumor size, the involvement of nearby structures, and other factors [19,20]. With optimally surgically treated localized disease, approximately 50% of high-grade STS patients eventually develop pulmonary metastases within five years [2,21–23]. Both neoadjuvant and adjuvant radiotherapy have reduced the local recurrences but were also associated with significant toxicity, especially in retroperitoneal STSs [24,25].

Metastatic STSs have very limited treatment options [26]. Systemic chemotherapeutic agents induce response in only 15–35% of the patients, irrespective of the histological subtype [27–29]. Doxorubicin represents the mainstay of treatment for decades and only small benefit was observed when combined with other chemotherapeutic agents [27–29]. The median survival of STSs patients after the administration of by chemotherapy is only 10–15 months [27–29].

Cancer immunotherapy has changed the treatment landscape in oncology, modified the therapeutic algorithms in multiple malignancies and, furthermore, became the leading treatment for metastatic diseases [30]. With the diverse immunotherapeutic approaches that are currently being applied, complete remissions have been observed in some patients [31–33]. However, a significant proportion of patients are immunotherapy resistant [34]. STSs belong to the tumors with only limited responses to immunotherapy [35,36].

While molecular characteristics of STSs are being urgently investigated among studies, the understanding of the events that occur within the tumor-immune system interplay in STSs are far from satisfactory [37]. The phenotypic profile of immune infiltrates of STSs should drive the process of decision-making whether to apply immunotherapy [34,38–40]. Furthermore, deciphering the immune cell signatures within the tumor may discriminate immunotherapy responders from non-responders and different immunotherapeutic approaches could be combined based on predominant cell subpopulations infiltrating the STS tumors [34,39,40].

In this review, we introduce the most important immune cell types infiltrating the STSs tumors and discuss different immunotherapies that would target these immune cells to enhance the anti-tumor immune responses and improve the prognosis of metastatic STSs patients.

## 2. Methods

A comprehensive review of the literature on diverse immune cell populations infiltrating the tumor microenvironment (TME) of STSs and a review of therapeutic approaches targeting these cell populations was conducted. Soft tissue sarcoma, T cells, CD8 T (cytotoxic) cells, CD4 T (helper) cells, natural killer (NK) cells, macrophages, T regulatory cells, Tregs, and FOXP3 T cells were used as the key words in the search strategy. The diagnosis of osteosarcoma, as well as Kaposi sarcoma, was excluded. Therapies targeting the particular immune cell population were searched through the official registry at clinicaltrials.gov and search databases. Excluded were clinical trials of unknown status and withdrawn clinical trials. Only English written and peer-reviewed articles published in indexed international journals until June 2021 were reviewed. Databases used for search included Medline/Pubmed, Scopus, and Web of Science. The selection process is summarized in Figure 1.
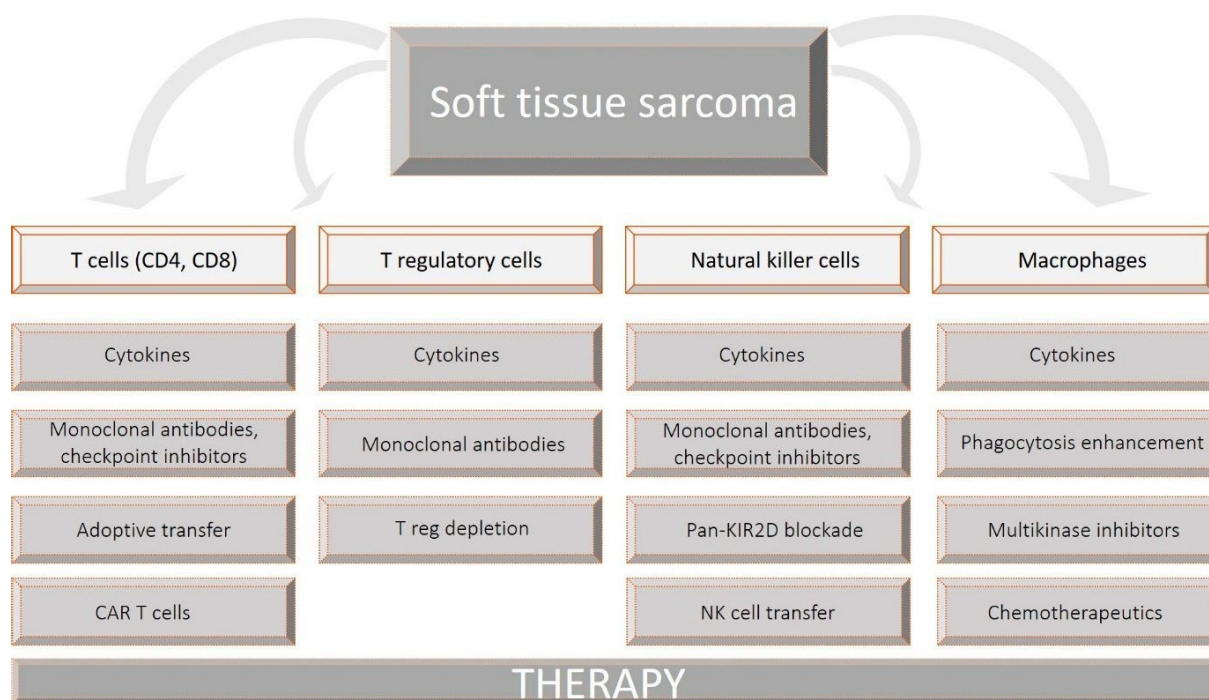
**Figure 1.** Scheme of the data selection process. Soft tissue sarcoma, T cells, CD3 T cells, CD8 T (cytotoxic) cells, CD4 T (helper) cells, natural killer (NK) cells, macrophages, T regulatory cells, Tregs, and FOXP3 T cells were used as the key words in the search strategy. Boxes below cell populations represent the search sub-categories. Therapies targeting the particular immune cell population were searched through the official registry at clinicaltrials.gov and search databases Medline/Pubmed, Scopus, and Web of Science. Articles published within the past ten years (Jan 1st 2011–July 1st 2021) were primarily taken into consideration.

### 3. T Cell Infiltration

T helper cells (CD4^{+}) and cytotoxic T cells (CD8^{+}) are the two main subpopulations of T cells that control and shape the immune responses in the tumor microenvironment (TME) [41].

T cells when activated through their T cell receptor (TCR) serve as the mediators of the adaptive immune response that efficiently migrate into the TME and induce cellular death in their target tumor cells [41,42]. The predominant cytotoxic mechanisms of CD8^{+} T cells include the production of death receptor ligands, such as Fas ligand or TRAIL; the production of perforin and granzyme; and the production of cytokines, such as TNF [43]. In most cancer types, the expression of immune checkpoint receptors, such as PD-1, CTLA-4, TIM-3, Lag-3, and other inhibitory molecules in CD8^{+} T cells is associated with the disease prognosis and is highly predictive of efficient immunotherapy with immune checkpoint inhibitors (CPIs) [44].

CD4^{+} T cells in the TME primarily serve as promotors of the executive functions of effector CD8^{+} T cells [45]. However, CD4^{+} T cells were also shown to bear cytolytic abilities and were proposed by a number of studies to represent the major anti-tumor T cell subpopulation [46]. CD4^{+} T cells differentiate into multiple cell sublineages, out of which Th1 cells are probably the most potent Th lineage against tumors [45].

Both cell populations, CD8^{+} and CD4^{+} T cells, also regulate the immune responses in the TME by secreting a broad range of cytokines Figure 2 [47].
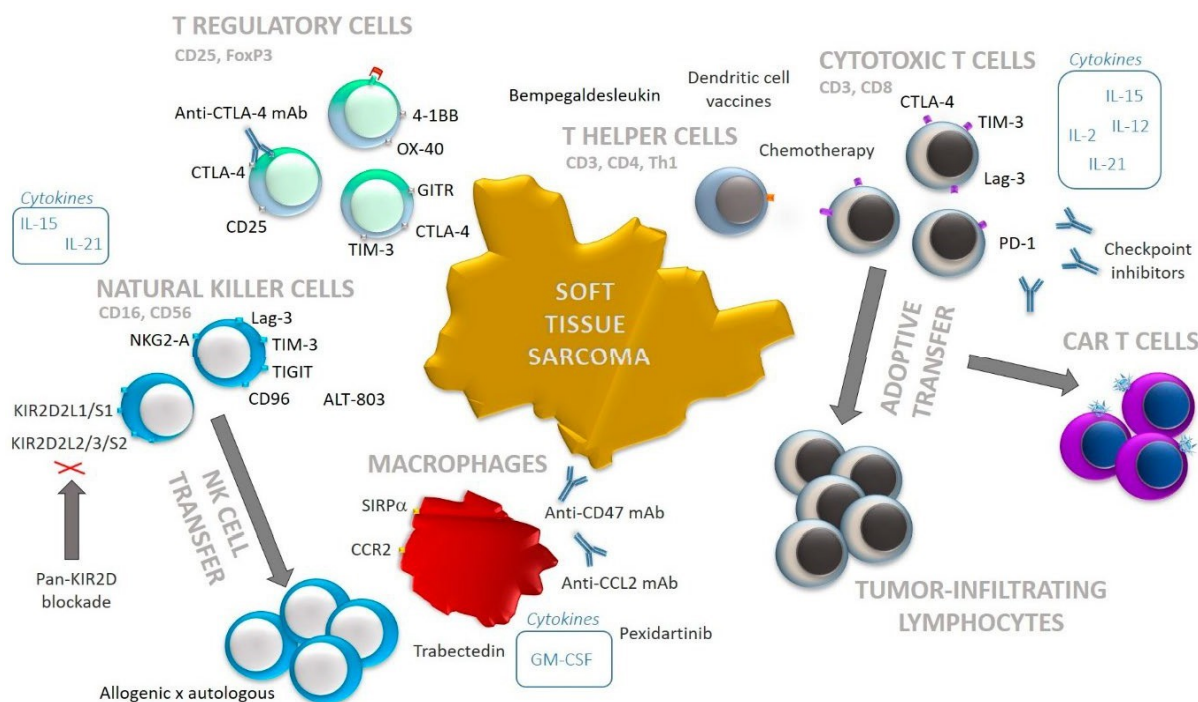
**Figure 2.** A schematic diagram for major cell populations infiltrating the tumor microenvironment of STSs and an overview of different therapies targeting these populations. Surface receptors can be targeted with monoclonal antibodies (mAbs), and diverse cytokines serve as potent promotors of cell functions. CD3$^+$ T cells and natural killer (NK) cells can be ex vivo expanded and adoptively transferred to a patient. Moreover, a generation of chimeric antigen receptor (CAR) T cells is allowed by a genetic modification of T cell receptor (TCR). Protumorigenic (M2) macrophages, as well as T regulatory cells, can be selectively depleted by synthetic agents or mAbs. The phagocytic capacity of macrophages, on the other hand, is secured by an administration of anti-CD47 mAbs. NK cells can be efficiently activated by a pan-KIR2D blockade together with IL-15 superagonist. Multiple immunotherapeutic approaches can be combined.

Different studies attempted to assess the proportions and phenotypes of T cells in STSs [48,49]. A recent study by Klaver et al. demonstrated that nearly one third of liposarcomas and leiomyosarcomas belong to the CD8$^+$ T cell-poor tumors, whereas pleomorphic sarcoma and myxofibrosarcoma were shown to have one of the highest infiltration with CD8$^+$ T cells [48]. Conversely, a study by Pollack et al. suggested that leiomyosarcomas belong to the inflammatory tumor types with high levels of T-cell-related gene expression, with several tumors demonstrating expression of PD-1 and very strong expression of PD-L1 [49]. However, studies evaluating the efficacy of anti-PD1 agents in the treatment of leiomyosarcomas were greatly disappointing [49]. Klaver et al. presented that only 7% of myxofibrosarcomas and 13% of pleomorphic sarcomas were poorly infiltrated with CD8$^+$ T cells [48]. Moreover, pleomorphic sarcomas and myxofibrosarcomas had also the highest fractions of PD-1$^+$CD8$^+$ T cells [48]. Interestingly, pleomorphic sarcoma displayed highest proportions of PD-1$^+$Lag-3$^+$TIM-3$^+$CD8$^+$ TILs, being comparable to malignant melanoma [48]. These results are also supported by a recent pooled analysis of anti-PD1 and anti-PD-L1 phase II clinical trials where undifferentiated pleomorphic sarcoma exhibited the highest response rates to treatment [50]. Another study by D'Angelo et al. showed that leiomyosarcoma, liposarcoma, synovial sarcoma and chondrosarcoma generally had low-density CD8$^+$ cells [51]. In another study, synovial sarcoma had significantly increased concentrations of CD8$^+$ TILs expressing PD-1 in metastatic tumors as compared to primary tumors [52].

Previous studies have discussed the association between the clinicopathologic factors and the infiltration of the TME with TILs [53–56]. It was also previously shown that one of the main challenges of successful immunotherapy is the inefficient T cell trafficking into the tumor tissue [34,57]. A recent study by Wustrack et al. has shown that in undifferentiated

pleomorphic sarcoma, larger tumors limited the immune infiltration with CD8^{+} T cells as the tumor size significantly correlated with a decrease in the frequency of CD8^{+} TILs [58]. Also, a high load of effector CD8^{+} T cells was associated with improved overall survival in these STSs [58]. According to the TME immunoprofiling among several different studies, undifferentiated pleomorphic sarcomas generally have a potential to respond to anti-PD-1 immunotherapy [35,58].

The assessment of PD-1 and PD-L1 expression status among STSs has been carried out by Movva et al. [59]. The report of 2000 sarcomas revealed over 50% of PD-1 and PD-L1 positive TILs in the TME of sarcomas [59]. However, both STSs and bone sarcomas were included into the study [59]. The PD-L1 expression was observed in 70% of undifferentiated pleomorphic sarcoma cases, in 75% of chondrosarcoma cases, in 77% of liposarcomas cases, but only in 32% of leiomyosarcomas cases [59].

Data regarding the proportions and roles of CD4^{+} T cells in the STS TME are limited and only little is known about their phenotypic patterns. Recently, authors Bi et al. have shown that the infiltration with CD4^{+} T cells positively correlated with better survival in STSs and could, thus, serve as a prognostic biomarker for STSs [60]. Moreover, in this study, CD4^{+} T cell infiltration levels were significantly associated to the overall survival in patients with undifferentiated pleomorphic sarcomas [60]. Another recent study showed the CD4^{+} T cell expression in leiomyosarcomas approximately 30% ($+/-22$%) but contained a large proportion of T regulatory cells (Tregs) while lacking the activation markers, such as CD69 and CD32 [61].

## 4. T Cell Immunotherapies in Soft Tissue Sarcomas

An immunotherapeutic approach that relies on the administration of stimulatory cytokines serves as an important regulator of T cell functions [62]. To date, the most commonly applied cytokine remains the interleukin-2 (IL-2) Figure 2 [62]. However, a careful balance must be achieved when selecting the optimal IL-2 concentration to avoid a preferential induction of CD4^{+}CD25^{+}Foxp3^{+} T regulatory cell (Treg) expansion [62]. Other cytokines, such as IL-12, IL-15, IL-21, and granulocyte macrophage colony-stimulating factor (GM-CSF), are still being evaluated in clinical trials [62].

In STSs, over 20 clinical trials have been initiated with the administration of IL-2 in a combination therapy. However, none of these trials have entered phase III of clinical testing (clinicaltrials.gov). A single-arm multi-cohort phase II study is currently ongoing with the aim to evaluate the immunological effectiveness and safety of IL-2 in combination with autologous dendritic cell vaccination (NCT04166006). Both approaches, IL-2 and DC vaccination should provide stimulatory signals to T cells and thus, promote the adaptive T-cell mediated immune responses in the TME [63,64]. Another phase II clinical trial in STSs is based on the administration of IL-2 in combination with autologous TILs and chemotherapy (NCT03449108) and the results are expected in 2022. A total of four clinical trials have been initiated with the IL-15, a potent activator of NK cells and T cells, but not Tregs [65]. Out of these phase I clinical trials, one trial is based on the administration of autologous activated T-cells expressing a second generation GD2 Chimeric Antigen Receptor (CAR), IL-15 and iCaspase9, and is currently recruiting (NCT03721068).

CPIs are blocking monoclonal antibodies (mAbs) targeting surface inhibitory receptors of T cells (Figure 2) [66]. To date, the most compelling results in clinical practice were observed with anti-CTLA-4 mAbs (ipilimumab, tremelimumab), anti-PD-1 mAbs (nivolumab, pembrolizumab, pidilizumab), and anti-PD-1-ligand mAbs (atezolizumab, avelumab, durvalumab) [67]. The efficacy of anti-PD-1 depends on its ability to restrain the TCR signaling pathway [68]. Anti-CTLA-4, on the other hand, is a competitive receptor that prevents binding of CD80/86 [69].

Response to immunotherapy with CPIs largely depends on the level of CD8^{+} TILs infiltration in the TME and on the expression of immune checkpoint molecules [34]. Since STSs are infiltrated with TILs in diverse proportions, many clinical trials with anti-PD-1, anti-PD-L1 and anti-CTLA4 have been initiated to distinguish the responders from non-

responders. To date, 48 clinical trials have been initiated in STSs patients with nivolumab (anti-PD-1), 39 with pembrolizumab (anti-PD-1), 30 with ipilimumab (anti-CTLA-4), 13 with atezolizumab (anti-PD-L1), 17 with durvalumab (anti-PD-L1), 9 with avelumab (anti-PD-L1), 5 with tremelimumab (anti CTLA-4), and 2 with cemiplimab (anti-PD-1) (clinicaltrials.gov). Only one CPI study has reached phase III of clinical testing and is currently recruiting (NCT04741438). In this French randomized prospective multicentre study, STSs patients will receive a combination of nivolumab (3 mg/kg) and ipilimumab (1 mg/kg) for a maximum treatment period 12 months (NCT04741438). The inclusion criteria cover patients with metastatic or unresectable advanced sarcomas of rare subtype.

Out of the most commonly applied CPIs, nivolumab, pembrolizumab, and ipilimumab entered phase II clinical trials with a great deal of promise (Table 1).

**Table 1.** A list of phase II and III active or recruiting clinical trials with nivolumab, pembrolizumab, and ipilimumab in soft tissue sarcomas. Excluded were withdrawn clinical trials, completed trials and trials of unknown status. Red triangle indicates phase III clinical trials.

**Phase II + III Clinical Trials**

| Drug Name | Diagnosis | Trial Design | Setting | CPI Dosage Regimen | Estimated Study Completion | Identifier |
|---|---|---|---|---|---|---|
| Nivolumab | Soft tissue sarcoma | Non-randomized | Combination therapy: Ipilimumab, Cryoablation | 3 mg/kg every 3 weeks x 4 doses. | October 2025 | NCT04118166 |
| Nivolumab | Soft tissue sarcoma | Randomized | Combination therapy: Relatlimab | 240 mg every 2 weeks | September 2024 | NCT04095208 |
| Nivolumab | Soft tissue sarcoma | Non-randomized | Combination therapy: Trabectedin | 240 mg every 3 weeks | October 2022 | NCT03590210 |
| Nivolumab | Soft tissue sarcoma | Randomized | Combination: Cabozantinib, Ipilimumab | 3 mg/kg every 3 weeks x 4 doses, followed by 480 mg every 4 weeks | January 2027 | NCT04551430 |
| Nivolumab | Sarcoma, Desmoid, Chondroma | Non-randomized | Combination: Trabectedin, Talimogene Laherparepvec | 240 mg every 2 weeks | December 2022 | NCT03886311 |
| Nivolumab | Advanced/metastatic sarcoma | Non-randomized | Combination: NKTR-214 | 360 mg every 3 weeks | September 2023 | NCT03282344 |
| Nivolumab | Advanced/metastatic sarcoma | Non-randomized | Combination: Trabectedin, Ipilimumab | 3 mg/kg every 2 weeks up to 26 doses | March 2022 | NCT03138161 |
| Nivolumab | Advanced/metastatic sarcoma | Non-randomized | Combination: Gemcitabine, Doxorubicin, Docetaxel | 240 mg IV on Day 1 of each cycle | December 2025 | NCT04535713 |
| Nivolumab | Soft tissue sarcoma | Non-randomized | Combination: Sunitinib | 240 mg every 2 weeks | September 2022 | NCT03277924 |
| Nivolumab | Recurrent/refractory sarcoma | Non-randomized | Monotherapy | 240 mg every 2 weeks | March 2029 | NCT03465592 |
| Nivolumab | Resectable or recurrent dedifferenti-ated/undifferentiated pleomorfic sarcoma | Randomized | A: monotherapy; B combination with Ipilimumab; C combination with RT; D combination with Ipilimumab and RT | IV on days 1, 15 and 29 in A, B; IV over 1 h on days 1, 15, 29 and 43 in C, D | October 2021 | NCT03307616 |

**Table 1.** *Cont.*

| Phase II + III Clinical Trials | | | | | | |
|---|---|---|---|---|---|---|
| **Drug Name** | **Diagnosis** | **Trial Design** | **Setting** | **CPI Dosage Regimen** | **Estimated Study Completion** | **Identifier** |
| Nivolumab | Angiosarcoma | Randomized | A: nivolumab, paclitaxel; B paclitaxel; C: nivolumab, cabozantinib S-malate | I.V. on Day 1 of each cycle, cycles repeat every 4 weeks | September 2023 | NCT04339738 |
| Nivolumab | Soft tissue sarcoma | Non-randomized | Combination: AL3818 | 240 mg every 2 weeks | December 2022 | NCT04165330 |
| Nivolumab | Epitheloid sarcoma | Non-randomized | Combination: Ipilimumab | Nivo and Ipi at predetermined dosage day 1 of a 21-day cycle for 4 cycles. | October 2025 | NCT04416568 |
| Nivolumab | Uterine sarcomas | Non-randomized | Monotherapy | 480 mg IV once every 4 weeks | August 2022 | NCT03241745 |
| Nivolumab | Soft tissue sarcoma | Non-randomized | Combination: BA3011 | Unspecified | January 2022 | NCT03425279 |
| Nivoluma | Sarcoma 2nd-line and relapsed/refractory | Non-randomized | Combination: NKTR-262, bempegaldesleukin | 360 mg every 3 weeks | December 2021 | NCT03435640 |
| Nivolumab | Leiomyosarcoma | Non-randomized | Combination: Rucaparib | 480 mg i.v. on day 1 of every four-week cycle | November 2022 | NCT04624178 |
| Nivolumab | Angiosarcoma, endometrial carcinosarcoma | Non-randomized | Monotherapy, Combination: Ipilimumab | Unspecified | August 2021 | NCT02834013 |
| Nivolumab | Advanced/metastatic sarcoma | Randomized | Combination: Ipilimumab | Nivolumab: 3 mg/kg i.v. every 2 weeks for 4 cycles; Ipilimumab 1 mg/kg IV over 60 min every 6 weeks for 4 cycles | August 2025 | ▲ NCT04741438 |
| Pembrolizumab | Advanced sarcoma | Non-randomized | Combination: Lenvatinib | 200 mg as a 30-min IV infusion, Q3W +/−3 days | March 2024 | NCT04784247 |
| Pembrolizumab | Advanced sarcoma | Non-randomized | Combination: Metronomic Cyclophosphamide | 200 mg every 3 weeks on day 8 for 3 weeks | August 2021 | NCT02406781 |

**Table 1.** *Cont.*

| Phase II + III Clinical Trials | | | | | | |
|---|---|---|---|---|---|---|
| Pembrolizumab | Soft tissue sarcoma of the Extremity | Randomized | Combination: Radiotherapy | 200 mg i.v. every 3 weeks | July 2025 | NCT03092323 |
| Pembrolizumab | Soft tissue sarcoma | Non-randomized | Combination: Axitinib | 200 mg i.v. infusion every 21 weeks, max up to 2 years | December 2022 | NCT02636725 |
| Pembrolizumab | Advanced/metastatic sarcoma | Non-randomized | Combination: Epacadostat | 200 mg/dose Day 1, Q 3 weeks | January 2022 | NCT03414229 |
| Pembrolizumab | Soft tissue sarcoma | Non-randomized | Combination: Eribulin | Pembrolizumab every 3 weeks | August 2024 | NCT03899805 |
| Pembrolizumab | Advanced/metastatic soft tissue sarcoma | Non-randomized | Combination: Doxorubicin | 200 mg intravenously every 3 weeks | February 2025 | NCT03056001 |
| Pembrolizumab | Soft tissue sarcoma | Non-randomized | Combination: Radiotherapy | i.v. every 3 weeks for 3 months | June 2023 | NCT03338959 |
| Pembrolizumab | Advanced/metastatic sarcoma | Non-randomized | Combination: Alimogene Laherparepvec (T-VEC) | Every 3 weeks | March 2022 | NCT03069378 |
| Pembrolizumab | Sarcoma of extremities | Non-randomized | Combination: Isolated Limb infusion (ILI) | 200 mg i.v. every 3 weeks | April 2023 | NCT04332874 |
| Pembrolizumab | Leiomyosarcoma and Undifferentiated Pleomorphic Sarcoma | Non-randomized | Combination: Gemcitabine | 200 mg every 3 weeks | December 2020 | NCT03123276 |
| Pembrolizumab | Advanced/metastatic Synovial Sarcoma | Non-randomized | Combination: Interferon gamma-1b | 200 mg i.v. every 3 weeks | April 2022 | NCT03063632 |
| Pembrolizumab | Soft tissue sarcoma | Non-randomized | Combination: Intra-tumoral BT-001 | 200 mg intravenously every 3 weeks | November 2024 | NCT04725331 |
| Pembrolizumab | Sarcoma | Non-randomized | Monotherapy | 200 mg i.v. every 3 weeks | December 2023 | NCT03012620 |
| Ipilimumab | Undifferentiated Pleomorphic Sarcoma Or Myxofibrosarcoma | Randomized | Combination: Envafolimab | 1 mg/kg every 3 weeks for a total of 4 doses | July 2022 | NCT04480502 |
| Ipilimumab | Soft tissue sarcoma | Non-randomized | Combination: Aldesleukin, nivolumab, fludarabine, cyclophosphamide | Unspecified | June 2024 | NCT03449108 |
| Ipilimumab | Sarcoma | Non-randomized | Combination: INT230-6 | Day 1 every 3 weeks for four treatments | July 2022 | NCT03058289 |

In an observational phase II clinical trial, pembrolizumab was administered in monotherapy to 42 STSs patients with diverse tumor histology (NCT02301039). In this study, one complete response was observed in a patient with undifferentiated pleomorphic sarcoma and a total of seven patients experienced objective responses [70]. To note, not a single leiomyosarcoma patient responded to the treatment with pembrolizumab [70]. Combination therapies with pembrolizumab include active and/or recruiting clinical trials with chemotherapy (NCT03123276), radiotherapy (NCT03338959), biologic therapy (NCT03126591), and tyrosine kinase inhibitors (NCT02636725).

Nivolumab is currently being evaluated for the treatment of STSs mostly in combination therapies (clinicaltrial.gov). Nivolumab monotherapy was tested in patients with locally advanced, unresectable, or metastatic sarcoma in a randomized multicenter, open-label phase II study (NCT02500797). In this study, only 5% of the patients receiving nivolumab responded to the treatment whereas addition of ipilimumab to nivolumab therapy led to an increased response rates highlighting a promising efficacy of the combination therapy [71].

In leiomyosarcomas patients, a phase II open label study is currently evaluating the effect of combination therapy with nivolumab and PARP inhibitor rucaparib (NCT04624178), and an interesting combination of nivolumab with trabectedin (macrophage affecting chemotherapeutic, see below) and oncolytic virus Talimogene Laherparepvec is to be evaluated in sarcoma patients in a currently recruiting phase II clinical trial (NCT03886311). Another phase I/II study in STSs patients aims to evaluate the efficacy and safety of nivolumab, together with ipilimumab and trabectedin as a first line treatment (NCT03138161). Nivolumab is further being evaluated in a combination therapy with chemotherapy (NCT04535713, NCT04339738), radiation therapy (NCT03307616), and targeted therapy (NCT03277924, NCT04416568).

Efficacy of ipilimumab therapy in STSs is being assessed in phase II clinical trials, including mostly combination therapies (Table 1). A single phase II clinical trial with ipilimumab monotherapy was carried out in patients with synovial sarcoma. However, the trial was terminated due to limited benefit of the treatment (NCT00140855).

ACT is a particular form of cell-based anticancer immunotherapy where both peripheral T cells and TILs can be used for ex vivo expansion and therapeutic administration to the patient [57]. This highly personalized therapy is to be studied in patients with advanced/metastatic STSs in a single center phase II open label study (NCT03725605). In this study, patients will be followed up for 15 months and the results are expected by 2023. In another phase II study, the efficacy of TIL infusion will be evaluated in a combination with chemotherapy in patients with multiple solid tumors, including STSs (NCT03935893). Appealing phase II study in synovial sarcoma patients is based on administering TBI-1301 (NY-ESO-1 specific TCR gene transduced autologous T lymphocytes) intravenously for 2 days following cyclophosphamide pre-treatment. Completion date is not yet estimated (NCT03250325).

A modification of traditional ACT is based on the infusion of the patients' ex vivo expanded T cells after previous genetic modification of T-cells to express a chimeric antigen receptor (CAR) specific for a tumor antigen [72]. This therapy is called CAR T cell therapy [72]. To date, four phase II clinical trials have been initiated with the application of CAR T cells in STSs. All studies are currently recruiting (clinicaltrials.gov; July 1st 2021).

## 5. Regulatory T Cell (Treg) Infiltration

Regulatory T cells (Tregs, CD4$^+$CD25$^+$FOXP3-expressing T cells) regulate and suppress other cell types of the immune system. Their modulatory functions include production of cytokines, expression of inhibitory molecules, cytolytic functions, disruption of Ca2$^+$ supply to the effector CD8$^+$ lymphocytes, and multiple other mechanisms causing T cell anergy [73]. In the TME, Tregs were found to promote the tumor growth by restraining effective anti-tumor immune responses [73]. In STSs, a recent study in 192 surgically-treated STSs patients has revealed that the presence of Tregs is associated with the increased risk

of local recurrence, irrespective of margins [74]. A study by D'Angelo et al. demonstrated that the proportions of FOXP3^{+} cells were relatively low as compared to CD4^{+} and CD8^{+} cells [51]. However, deep tumors were more likely to be associated with high FOXP3^{+} infiltration while superficial tumors had relatively low FOXP3^{+} infiltration [51]. A study by Klaver et al. further showed that pleomorphic sarcoma had a significantly lower fraction of Tregs as compared to leiomyosarcoma [48]. Moreover, Keung et al. observed that in patients with undifferentiated pleomorphic sarcoma undergoing neoadjuvant radiotherapy, tumors had an increased median number of Tregs which provided a rationale for a combination of radiotherapy and CPIs [75]. In a study by Que et al., both proportions of Tregs and the PD-L1 expression status were evaluated among 163 STSs patients [76]. In this study, a high load of Tregs positively correlated with the tumor stage, tumor grade and the depth of invasion. Also, PD-L1 expression, FOXP3^{+} Tregs infiltration and PD-L1/FOXP3 were significantly associated with overall survival in patients with undifferentiated pleomorphic sarcoma [76]. Since both PD-L1 and FOXP3 were highly expressed STS patients with poor prognosis, the authors call for combination of Treg depletion therapy and CPIs [76].

## 6. Targeting Tregs in Soft Tissue Sarcoma

The idea of blocking the immunosuppressive functions of Tregs has raised many concerns [77]. Principally, as Tregs ensure optimal immune responses to prevent autoimmunity, blocking their effector functions might result in a severe immune dysregulation [77].

Treg depletion for the purpose of enhancing antitumor immune responses has been previously tested mostly by an administration of Treg-specific cell-depleting antibodies. The target molecules included CD25, CTLA-4, GITR, 4-1BB, OX-40 and other molecules in diverse cancer types [77], Figure 2.

Molecule CD25 has a crucial role in the development and activation of T regs [73]. CD25 is a component of the high-affinity heterotrimeric IL-2 receptor that has been widely studied in the context of cancer [73]. Several issues, however, limit the wide use of anti-CD25 mAb [73]. Most importantly, effector T cells also share CD25 receptor making it difficult to selectively deplete Treg cells [78]. Therapeutic targeting of CD25 has been shown in metastatic melanoma patients to significantly deplete Tregs without impairing CD8^{+} T cell functions [78]. In STSs, a single clinical trial attempted to evaluate the efficacy of adoptive transfer with CD25 depleted autologous lymphocytes in rhabdomyosarcoma. Data are not yet available (NCT00923351).

Bempegaldesleukin is a recombinant form of human IL-2 that serves as a CD122-preferential IL-2 pathway agonist [79]. Hence, Bempegaldesleukin promotes proliferation of CD8^{+} T cells and NK cells without enhancing Treg activation [79]. A phase I/II study of bempegaldesleukin in combination with nivolumab has been initiated to evaluate the efficacy and safety in patients with recurrent or refractory malignancies, including rhabdomyosarcoma (NCT04730349).

CTLA-4 is constitutively expressed on T regs. A recent study by Zappasodi et al. has demonstrated that CTLA-4 blockade drives loss of Treg stability in glycolysis-low tumors [80]. Moreover, the anti-CTLA-4 therapy led to an increased IFN expression in the remaining Tregs and, therefore, attacked tumors at multiple levels [80]. Several clinical trials are currently ongoing with anti-CTLA-4 mAbs (see above). To date, clinical trials targeting different Treg molecules, such as GITR or OX-40 have not been initiated in STSs.

## 7. Natural Killer (NK) Cell Infiltration

NK cells belong to the family of innate lymphoid cells (ILC) and, in the context of cancer, NK cells are believed to be the main effector cells of the innate immune system [81]. NK cells are similarly to CD8^{+} T cells highly cytotoxic with the ability to produce proapoptotic Fas ligand and TRAIL, as well as the perforins and granzymes [81,82]. NK cells, are not only capable of triggering apoptosis in malignant cells but also shape the TME by a secretion of large amounts of cytokines [83]. Previous reports have also shown that NK cells prevent metastases by eliminating circulating tumor cells [84]. In STSs, the presence

of NK cells has been reported through a limited number of studies. Sorbye et al. reported intratumoral NK cell status in 249 patients. In this study, a tendency towards improved overall survival was observed in STSs patients with high loads of CD57$^+$ NK cells in the peritumoral area [85].

Bücklein et al. have provided a detailed analysis of NK cell presence and function in STSs patients and, unlike in other malignancies, peripheral blood NK cells exhibited a profound impairment in cell function, especially in the intermediate and high-grade STSs [86]. Judge et al. further demonstrated that intratumoral NK cells express more activation and exhaustion markers as compared to peripheral blood NK cells [87]. Furthermore, ex vivo stimulation with IL-15 further increased both activation and exhaustion markers in intratumoral and peripheral blood NK cells [87]. The authors observed a significant upregulation of TIGIT receptor and, therefore, suggested a therapeutic potential of TIGIT blockade together with IL-15 therapy [87].

## 8. NK Cell-Based Immunotherapies in Soft Tissue Sarcomas

IL-15 is a potent activator of NK cells with a major importance for NK cell proliferation and survival [88]. The advantages of IL-15 over IL-2 treatment include lower toxicity and lack of T-reg cell induction [89]. A phase I clinical trial evaluating the anti-tumor actions of IL-15 is administering autologous ex vivo activated NK cells with or without recombinant IL-15 to patients with solid tumors, including sarcomas (NCT01875601). Similarly, IL-15 is to be utilized as a compound of CAR T cells in a launching clinical trial with rhabdomyosarcoma and liposarcoma patients. In this trial, IL-15 is believed to deliver superior efficacy over classic CAR T cells. The genetic construct is called AGAR T cells (NCT04377932).

NK cells are known for their expression of a wide variety of activation and inhibitory receptors [90]. NKG2A is highly expressed on NK cells and transmits inhibitory signal [90], Figure 2. Anti-NKG2A antibody, monalizumab, is currently being tested among 13 different clinical trials, none of which is focused on STSs (clinicaltrials.gov, July 1st 2021).

Another NK receptor targeting agent is lirilumab, a pan-KIR2D blocker, which also has no ongoing clinical trials in STSs [91]. TIM-3 and Lag-3 are inhibitory checkpoint molecules with high expression in NK cells [92]. Although TIM-3 mAbs are evaluated in preclinical and phase-I clinical trials, data on their efficacy in STSs are lacking [93]. It should be noted that twelve anti-TIM-3 clinical trials have already been initiated (clinicaltrials.gov, July 1st 2021).TIGIT and CD96 are other NK cell receptors that serve as an optimal target for NK-mediated cancer immunotherapy [94]. While CD96 affects mainly the cytokine production of NK cells, TIGIT allows direct inhibition of NK cytotoxic functions through its ITIM domain [95]. Several clinical trials aimed at targeting TIGIT signaling pathway are currently underway, however, the efficacy of anti-TIGIT antibodies in STSs has not yet been evaluated [96]. Anti-CD96 therapy is still mostly under consideration in pre-clinical studies [94].

Adoptive cell transfer of NK cells has also become a promising treatment approach for advanced and/or metastatic diseases [97]. A phase II clinical trial in adult STSs evaluated the efficacy of cryosurgery in combination with NK cell immunotherapy. In this study, NK transfusions were given intravenously in three different time points with each infusion containing 8–10 billion cells (NCT02849366). Another pilot study aimed to evaluate the efficacy of intravenous infusion of expanded, activated haploidentical NK Cells (NCT02409576). NK cells were administered together with chemotherapy, radiotherapy, and cytokine support with IL-2 cytokine. A study combining NK cell infusions together with ALT-803, which is a pharmacological IL-15 superagonist is based on NK cells from non-HLA matched donors (NCT02890758). Even though, allogenic NK cell infusions generally showed poor anti-tumor activity in clinical trials, the combination with ALT-803 could significantly enhance the NK cell cytotoxicity and finally provide desirable responses in STSs [98].

## 9. Macrophages

Macrophages differentiated from circulating monocytes that efficiently migrate within the tissue and continue trafficking into the TME are called tumor-associated macrophages (TAMs) [99]. TAMs are capable of phagocytosis, as well as regulation of the tissue growth and repair [99]. With the ability to produce immunosuppressive cytokines, such as IL-10 and TGF-β, to upregulate Tregs and to promote Th2 immune response, TAMs have become major contributors to the cancer progression [100]. However, it is the TME and the tumor-derived signals that intensify TAM functions [100].

In different cancer types, TAMs were associated with poor prognosis and reduced overall survival (OS) [101]. Even though TAMs mainly exploit protumorigenic mechanisms, several antitumor functions of TAMs have also been reported [102].

In a recent study by Dancsok et al., TAMs were investigated in 1242 sarcoma specimens [103]. The study revealed that across nearly all sarcoma types, macrophages outnumber TILs and thus dominate the immune landscape of STSs [103]. In this study, TAMs were most frequently observed in pleomorphic sarcomas, such as undifferentiated pleomorphic sarcoma and dedifferentiated liposarcomas [103]. As opposed, the lowest macrophage infiltration was found in synovial sarcoma, myxoid liposarcoma, clear cell sarcoma, and low-grade fibromyxoid sarcoma [103]. In synovial sarcoma, another study also reported that lower TAM infiltration is associated with better overall survival [104]. A recent study by Tsagozis et al. has highlighted that the immune cells infiltrating STSs are poorly characterized to date [105]. Therefore, the authors provided an immunohistochemical analysis of STSs tumors with different histology and, in accordance with the study by Dancsok et al., also found much higher densities of TAMs as compared to TILs in the sarcoma TME [103,105]. In this study, the pan-macrophage marker CD68 correlated with high immune cell infiltration in general and most macrophages were M2-polarized [105]. In a study by Shailaja et al., specific alteration in TAM densities were observed in STSs patients responding to neoadjuvant chemotherapy, suggesting that chemotherapy does indeed significantly modulate the TME of STSs [106].

In leiomyosarcoma, authors Lee et al. described a significant association between high density of TAMs and worse disease-specific survival [107]. This was further supported by another study correlating the high load of TAMs with poor survival in leiomyosarcomas [108]. Undifferentiated pleomorphic sarcoma was already reported to have high levels of macrophage infiltration [103]. A study by Shiraishi et al. further revealed that a high percentage of TAMs is related to shortened overall survival and to high AJCC stage and high FNCLCC grade [109]. This is particularly important since tumor grade is the most important prognostic factor for STSs to date [9]. Similar to undifferentiated pleomorphic sarcoma and leiomyosarcoma, myxoid liposarcoma was also shown to be infiltrated with M2 macrophages that negatively determine the patients' outcome and thus, remain a candidate for macrophage-targeted therapies [103,110].

## 10. Therapies Targeting Macrophages

Since macrophages represent the predominant cell type in most STSs, different clinical trials have been initiated to either suppress the pro-tumorigenic functions of TAMs or to modulate the natural ability of TAMs to eliminate target cells [111].

CD47 is a transmembrane protein that is highly expressed on neoplastically transformed cells [112]. CD47 binds to SIRPα, and this receptor-ligand interaction releases "don't eat me" signals that inhibit macrophage-mediated phagocytosis (Figure 2) [112]. Despite promising pre-clinical studies with anti-CD47 mAbs promoting the macrophage-mediated phagocytosis towards malignant cells of leiomyosarcoma, clinical trials in human STSs have not yet been carried out [113]. A phase I clinical trial administering an antibody-drug conjugate SGN-CD47M in patients with various solid tumors, including STSs, was terminated in 2020 due to the sponsors' decision (NCT03957096).

A multikinase inhibitor Pexidartinib (PLX3397) is currently being evaluated in a phase I/II clinical trial for the treatment of multiple sarcomas including liposarcoma,

leiomyosarcoma, synovial sarcoma, and rhabdomyosarcoma (NCT02584647). Pexidartinib is an inhibitor of proto-oncogene receptor tyrosine kinase (KIT), colony-stimulating factor-1 receptor (CSF1R) and FMS-like tyrosine kinase 3 (FLT3), and was shown to decrease the load of intratumoral TAMs and increase the proportions of CD4^{+} and CD8^{+} T cells, thus contributing to the tumor regression [114]. Due to diverse therapeutic targets of pexidartinib, its role in targeting macrophages include limiting their differentiation, extravasation, and polarization towards M2 phenotype [114,115]. Results of the first clinical trials in sarcoma tumors are eagerly awaited.

Trabectedin is a chemotherapeutic drug which was recently approved in the treatment of advanced STSs [116]. Trabectedin is classified as an alkylating drug, as it interferes with the cell division and the DNA repair. However, previous studies have also highlighted the ability of trabectedin to deplete both circulating monocytes and TAMs [117]. The selectivity of trabectedin against mononuclear phagocytes was explained as a result of rapid activation of caspase 8 by membrane signaling TRAIL receptors which are highly expressed in monocytes/macrophages [117]. Since most STSs are primary chemotherapy resistant, it may be the macrophage depletion that lies behind the efficacy of trabectedin in STSs [117,118].

Other possible therapeutic targets include chemokine and chemokine receptors regulating the macrophage trafficking towards the tumor [119]. CCL2-CCR2 axis serves as the major macrophage chemoattractant and has been proposed a suitable therapeutic target by previous studies [120]. Clinical trials with chemokine blockade in STSs are lacking but urgently needed to open novel options in the treatment of STSs [120].

## 11. Discussion

Soft tissue sarcomas are rare but mostly lethal mesenchymal tumors [4]. STSs are extremely biologically and clinically diverse tumors with more than 80 histological subtypes [2,3]. Due to such rarity of these tumors, clinical trials often evaluate all STSs types together, which may contribute to the mixed results from early immunotherapy clinical trials [103,121]. Localized STSs can be effectively treated by surgery with a five-year relative survival rate of 81% (STS statistics, Cancer.net, ASCO). However, the treatment of metastatic STSs has not changed for decades and, with the current conventional therapies, only small chances for the improvement in the overall survival rates are secured [4]. The TME of STSs tumors is infiltrated by diverse proportions of immune cells which provides a rationale to stratify patient according to the TME immune parameters [4,48]. CD8^{+} T cells belong to the main antitumor players in the TME, the infiltration of the TME with CD8^{+} T cells, and the expression of immune checkpoint molecules have become prerequisites for an effective immunotherapy with CPIs [122]. Pleomorphic sarcoma and myxofibrosarcoma were shown to have one of the highest infiltration with CD8^{+} T cells and the highest expression of PD-1 [48]. Therefore, these tumors are expected to equally benefit from CPIs [48]. In addition, pleomorphic sarcoma displayed a highly comparable immune landscape to malignant melanoma, suggesting an optimal suitability for immunotherapeutic approaches [48]. Interestingly, in larger tumors, CD8^{+} T cells tended to become excluded from the TME which could imply a limited efficacy of CPIs in tumors of greater size [58]. Leiomyosarcomas and liposarcomas have generally low CD8^{+} T cell infiltration among different studies which is reflected in the greatly disappointing results in clinical trials [71]. Out of T cell immunotherapies, CPIs are the most widely used agents. However, both nivolumab and ipilimumab in monotherapies showed only limited efficacy [71,123]. The undifferentiated pleomorphic sarcoma histology is associated with better response and CPIs combination therapies, such as nivolumab plus pembrolizumab, significantly increase the response rates in STSs patients. Other promising clinical trials include the ACT or CAR T cell therapy [124].

Poor immunotherapy responses in patients with leiomyosarcomas may also be associated with their relatively high loads of Tregs as compared to pleomorphic sarcomas [48]. TME infiltration with Tregs positively correlates with the tumor grade, stage, and the depth

of invasion [76]. Moreover, in undifferentiated pleomorphic sarcomas, Treg infiltration increases after neoadjuvant radiotherapy and affects the overall survival of patients [76]. Blocking the immunosuppressive functions of Tregs, however, raises many safety concerns and, to date, mostly anti-CTLA-4 agents show a great deal of promise in affecting both CD8$^+$ T cells in addition to Tregs [77,80]. Data regarding the presence and phenotypes of NK cells in STSs are quite limited. Hence, detailed analyses would be truly beneficial in STSs. While intratumoral NK cells of STSs exhibit numerous activation and exhaustion markers, peripheral NK cells in STS patients were observed as functionally impaired [86,87]. Patients undergoing NK-cell based immunotherapies mostly profit from administration of IL-15, a potent NK cell activator, while anti-NKG2A therapy is still lacking among clinical trials in STSs [125,126]. Macrophages dominate the immune landscape of STSs and are associated with the tumor grade and outnumber TILs across nearly all sarcoma types [103]. TAMs are frequently observed in pleiomorphic sarcomas, while the infiltration among liposarcomas, clear cell sarcomas, and synovial sarcomas is relatively low. It should be noted that alterations in TAM densities were shown in patients after neoadjuvant chemotherapy [106]. Therapies targeting macrophages are thus very promising in STSs. Preclinical studies reveal the potential of anti-CD47 therapy for leiomyosarcoma, and clinical trials employ mostly pexidarinib or trabectedin [103,120].

With the current knowledge of diverse immune cell populations infiltrating STS tumors, the ongoing clinical practice could fundamentally change and allow stratification of patients based on the immune landscape of the TME. As distinct histotypes of STSs are recognized to be infiltrated with immune cells in diverse proportions and are only sensitive to specific cytotoxic drugs, it is likely that also preclinical research will focus on deciphering the optimal histology-driven therapeutic regimens.

It is already clear that clinical trials aiming at multiple immune cell populations, such as T cells and macrophages that are triggering both the innate and adaptive immunity, bring a great deal of promise. As shown in clinical trials, immunotherapies can be easily combined and chemotherapy can serve as a powerful tool to sensitize TME to immunotherapy in these mostly chemoresistant tumors.

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## 4.3 „A novel anti-CD47-targeted blockade promotes immune activation in human soft tissue sarcoma but does not potentiate anti-PD-1 blockade"

Většina pacientů s měkkotkáňovým sarkomem stále umírá na rozvoj metastáz do několika let od počáteční diagnózy. Systémová chemoterapeutika vyvolávají odpověď pouze u 15–35 % pacientů a neoadjuvantní/adjuvantní radioterapie sice snižuje pravděpodobnost lokální recidivy, na druhou stranu bývá spojena se značnou toxicitou. Navíc kupříkladu u retroperitoneálních STS bývá často problematické podání adekvátní terapeutické dávky. Podíváme-li se na jiné léčebné modality, imunoterapie prostřednictvím checkpoint inhibitorů zatím neprokázala přesvědčivé výsledky u STS, ač některé histologické podtypy jistý benefit z terapie prokázaly. Jedním z hlavních důvodů, který stojí za relativně nízkou terapeutickou odpovědí checkpoint inhibitorů u STS, může být relativně nízká infiltrace nádorů imunitními T-buňkami. Na druhou stranu byla prokázána značná přítomnost makrofágů v nádorovém mikroprostředí STS, což značí potenciál v léčbě cílené právě na tuto imunitní populaci.

V této studii jsme hodnotili potenciální synergické účinky kombinace monoklonálních protilátek anti-PD-1 a anti-CD47. Efektivitu léčby jsme hodnotili in vitro prostřednictvím měření aktivace imunitních buněk ve smyslu produkce cytotoxických cytokinů. Pozorovali jsme, že po makrofázích jsou další nejčastější buněčnou populací v STS CD8 T-buňky. Vysoký podíl makrofágů a T-buněk nás dále vedl ke zkoumání

kombinované aktivace T-buněk a makrofágů prostřednictvím podání anti-PD-1 a anti-CD47.

Naše zjištění ukázala, že jak anti-PD-1, tak anti-CD47 terapie je schopna signifikantně zvýšit produkci prozánětlivých cytokinů IL-2, IFN-$\gamma$ a TNF-$\alpha$ in vitro. Nicméně překvapivým zjištěním bylo, že společné podávání anti-CD47 a anti-PD-1 nebylo účinnější, naopak vedlo k potlačení produkce cytotoxických cytokinů, a to i při dělení kohorty dle různých parametrů.

V naší studii byla nejvýznamnější odpověď na anti-CD47 terapii pozorována u nediferencovaného pleomorfního sarkomu, což byl také histologický podtyp s nejvyšší expresí CD47. Na druhou stranu, CD47 byl široce exprimován i v jiných podtypech STS, jako jsou high-grade myxofibrosarkomy nebo angiosarkomy.

Naše data naznačují, že kombinované strategie založené na checkpoint inhibitorech a anti-CD47 blokádě nemusejí zajistit požadovaný výsledek. Obě terapie, buď anti-PD-1, nebo anti-CD47 samostatně, by však mohly mít terapeutický potenciál u vybraných nádorů po pečlivém vyhodnocení CD47 a PD-1 exprese v nádoru.

**RESEARCH**

# A novel anti-CD47-targeted blockade promotes immune activation in human soft tissue sarcoma but does not potentiate anti-PD-1 blockade

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## Abstract

**Purpose** The treatment options for metastatic soft tissue sarcomas (STSs) are limited. In most cases, immunotherapy with immune checkpoint inhibitors has not been successful so far. Macrophages dominate the immune landscape of STSs; thus, combinatorial strategies aiming at both tumor-infiltrating lymphocytes and macrophages may represent a particularly relevant treatment approach for metastatic or recurrent STSs.

**Methods** In this cohort study, 66 patients who underwent surgery for STSs were enrolled. Tumor cells and tumor-infiltrating immune cells were analyzed using flow cytometry and immunohistochemistry. In cell suspensions obtained from surgical resections, human T cells were activated by superparamagnetic polymer beads and cultured at a concentration of $0.3 \times 10^6$/ µl in the absence or presence of therapeutic monoclonal antibodies (anti-PD-1, anti-CD47, and anti-PD-1 + anti-CD47). Supernatants from cell suspensions were analyzed using multiplex Luminex cytokine bead-based immunoassays.

**Results** The most profound response to anti-CD47 therapy was observed in an undifferentiated pleiomorphic sarcoma which also displayed high expression of CD47 in the tumor microenvironment. Both anti-PD-1 and anti-CD47 therapies drastically increased the production of pro-inflammatory cytokines in the tumor microenvironment of STSs, but co-administration of both agents did not further increase cytokine secretion. Furthermore, all patient samples treated with a combination of both anti-PD-1 and anti-CD47 antibodies showed a dramatic reduction in cytokine secretion.

**Conclusion** Our findings suggest that anti-PD-1 and anti-CD47 therapies do not enhance each other, and the combined application of anti-PD-1 and anti-CD47 agents in vitro limits rather than potentiates their efficacy.

**Keywords** Immune checkpoint inhibitor sarcoma · Combined immunotherapy · Don't eat me signal · Leiomyosarcoma · Undifferentiated pleiomorphic sarcoma

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## Introduction

Soft tissue sarcomas (STSs) are malignant mesenchymal tumors with highly heterogeneous clinical presentation, biological behavior, and histological structure (Gamboa et al. 2020). With more than 80 histological subtypes of STSs, studies defining robust prognostic or therapeutic biomarkers are lacking. Surgery remains the most important treatment option for patients with localized STS (Ozaniak et al. 2021; Spolverato et al. 2020). However, even with the major improvements in local control rates, metastases and death occur in 50% of patients diagnosed with high-risk STSs (Cormier and Pollock 2004; Kawamoto et al. 2020).

In patients with STSs, the lungs are the most common site of generalization (Digesu et al. 2016). For metastatic disease, the front-line therapy consists of anthracycline-based chemotherapy (Singhi et al. 2018). However, the efficacy of chemotherapy in STSs varies, with most histological subtypes showing only very limited chemosensitivity (Banerji and Kanjilal 2005).

For that reason, a detailed search for novel therapeutic options, including combinatorial strategies, is urgently needed in STSs. These novel therapies have the potential to rapidly change the course of the disease, offering success rates comparable to those following the introduction of tyrosine kinase inhibitors for the treatment of gastrointestinal stromal tumors or the introduction of immunotherapy for the treatment of metastatic melanoma (Songdej and von Mehren 2014; Weiss et al. 2019).

CD47 is a transmembrane protein that binds to the ligands signal regulatory protein (SIRP)α, thrombospondin (TSP)-1, and integrins αvβ3 and α2β1. CD47 is widely expressed in human cells; however, the expression in tumor cells is significantly higher (Huang et al. 2020). The CD47-SIRPα signaling pathway triggers a "don't eat me" signal that inhibits macrophage-mediated phagocytosis; thus, tumor cells overexpress CD47 to limit the anti-tumor activities of phagocytic immune cells (Huang et al. 2020; Strizova et al. 2020). CD47 overexpression has been described in multiple cancers, including chondromas and angiosarcomas with up to 80% of cells expressing the CD47 molecule (Dancsok et al. 2020; Jiang et al. 2021; Zhang et al. 2020). With a great deal of promise shown in preclinical trials, therapeutic strategies targeting CD47 have entered phase I/II clinical trials in patients with various solid tumors (Jiang et al. 2021).

The success of anti-CD47 therapy critically depends on the composition of the tumor microenvironment (TME) (Vonderheide 2015). The TMEs of STSs are determined by a variety of different cells, including tumor cells, extracellular matrix cells, and tumor-infiltrating immune cells (TIICs), such as phagocytic cells, antigen-presenting cells, natural killer cells, and T cells (Raj et al. 2018; Zhu and Hou 2020).

The infiltration of the TME by macrophages is an important factor for anti-CD47 therapy efficacy (Weiskopf et al. 2016) Recent studies have demonstrated that across nearly all sarcoma types, macrophages dominate the immune landscape of STSs (Dancsok et al. 2020). Moreover, macrophages in STSs largely outnumber tumor-infiltrating lymphocytes (TILs) (Dancsok et al. 2020).

To date, most studies have evaluated the effect of CD47 blockade in promoting innate phagocyte-mediated immunity against cancer (Chao et al. 2010; Kim et al. 2012; Weiskopf et al. 2016). Impact on T cells is a far less explored area of research (McCracken et al. 2015). While enhanced T-cell responses observed in anti-CD47 therapy have been generally attributed to increased phagocytosis by antigen-presenting cells and their presentation of tumor antigens to T cells, other studies have shown that blockade of CD47 can activate T-cell cytotoxicity directly and facilitate the cytolytic activity of CD8$^+$ T-cells (Soto-Pantoja et al. 2014; Tseng et al. 2013). Moreover, in fibrosarcoma models, blockade of CD47 was also proven to enhance the recruitment of CD8$^+$ T cells (Soto-Pantoja et al. 2014).

Since TILs are the major target of the most successful immunotherapies, combinatorial strategies aiming at both TILs and macrophages might represent a particularly relevant treatment approach for metastatic or recurrent STSs (Li et al. 2020).

In the current study, we evaluated the potential additive effects of combining anti-PD-1 and anti-CD47 therapies on anti-tumor responses in human STSs.

## Materials and methods

### Patients and tumor samples

A total of 66 patients who underwent surgery for STSs between January 2019 and June 2021 were enrolled in this study, and 73 tissue samples were analyzed. The female:male ratio was 1:1. The mean age was 63.5 years and ranged from 24 to 90 years. Out of 66 patients, 54 were admitted and operated on for localized disease, and three patients received neoadjuvant treatment. The CD47 expression status in patients with neoadjuvant treatment ranged from 0 to 3 and did not statistically differ from that in the rest of the study cohort. Another three patients, whose CD47 expression levels in tumor tissues were analyzed, were also included in subsequent Luminex and flow cytometry analyses. Furthermore, in four patients, both primary tumors and metastatic lesions occurring after $\geq$ 12 months were screened for CD47 expression. However, the CD47 expression levels of the primary tumors correlated with those of the metastatic lesions in these four surgically treated patients. The clinicopathological characteristics of the study participants

**Table 1** Characteristics of the study cohort

| Variable | Patients (n) | Patients (%) |
|---|---|---|
| Age (in years) | | |
| > 75 | 35 | 53.00 |
| 75–65 | 16 | 24.20 |
| < 65 | 15 | 22.70 |
| Sex | | |
| Male | 33 | 50.00 |
| Female | 33 | 50.00 |
| Grade | | |
| Low | 11 | 16.70 |
| High | 55 | 83.30 |
| Histology | | |
| Undifferentiated pleiomorphic sarcoma | 18.00 | 27.3 |
| Dedifferentiated liposarcoma | 11.00 | 16.70 |
| Pleiomorphic liposarcoma | 5.00 | 7.60 |
| Myxoid liposarcoma | 4.00 | 6.10 |
| Well-differentiated liposarcoma | 6.00 | 9.10 |
| Myxofibrosarcoma | 11.00 | 16.70 |
| Alveolar sarcoma | 2.00 | 3.00 |
| Leiomyosarcoma | 2.00 | 3.00 |
| Pleiomorphic rhabdomyosarcoma | 2.00 | 3.00 |
| Chondrosarcoma | 2.00 | 3.00 |
| Angiosarcoma | 1.00 | 1.50 |
| Spindle cell sarcoma | 1.00 | 1.50 |
| Sarcoma not otherwise specified | 1.00 | 1.50 |

are summarized in Table 1. The study was conducted ethically following the World Medical Association Declaration of Helsinki. All patients provided written consent to participate in the study, and the study was approved by the Ethics Committee for Multi-Centric Clinical Trials of the University Hospital Motol (Reference no.: EK-189/20). The experimental design of the study is shown in Fig. 1.

## Immunohistochemistry

Formalin-fixed paraffin-embedded tissue samples ($n = 61$) were retrospectively retrieved, and 3-µm-thick sections were stained for the presence of CD47 molecules using prediluted antibodies. Polyclonal anti-CD47 antibody (PA5-80435; Thermo Fisher Scientific, Massachusetts, USA) was used for the detection of CD47 in tumor cells, and each slide was scored manually by an experienced pathologist. Cytoplasmic and membranous stainings of tumor cells were considered positive (Fig. 2). For each case, the extent of CD47 staining was assessed and graded using a scale of 0–3:

0: no staining of tumor cells (negative).

1 + : less than 1/3 staining of the total tumor area (focal positivity).

2 + : 1/3–2/3 staining of the total tumor area (moderate positivity).

3 + : more than 2/3 staining of the total tumor area (diffuse positivity).

## TIIC isolation and single-cell suspension protocol

TIICs were isolated from surgical resections performed at Motol University Hospital, Prague, with the approval of the Ethics Committee. Tissue samples were placed on a sterile 8-well plate, and each sample was mechanically dissociated into small pieces using two single-edged razor blades. Next, the obtained tissue pieces (approximately 1 mm in diameter) were incubated in 5 ml of RPMI-1640 medium (Gibco™ RPMI 1640 Medium, Thermo Fisher Scientific) at room temperature (RT). The tissue was enzymatically digested by collagenase type IV and DNAse I for 30 min in a $CO_2$ incubator. After 30 min, the single-cell suspension was filtered through a 70-µm nylon strainer and washed in phosphate-buffered saline (PBS). To remove red blood cells, the cell suspension was then supplemented with Ammonium-Chloride-Potassium Lysing Buffer (Thermo Fisher Scientific) for 10–15 min at RT. After centrifugation ($500 \times g$, 10 min, RT), the pelleted cells were resuspended in cold (4 °C) PBS containing 2 mM EDTA.

## Flow cytometry

Flow cytometry was used as a complementary method to immunohistochemistry to characterize the proportions of tumor-infiltrating cells in vitro. The isolated cells were stained after a 30 min incubation with fluorophore-conjugated protein-specific antibodies according to the manufacturer´s instructions and recommendations. The following monoclonal antibodies (mAbs) were used: CD45 A700, CD4 Pacific Blue, and CD8 PeDy 594 (all Exbio, Prague, Czech Republic). The isolated cells were then washed and analyzed using a BD LSRFortessa flow cytometer (Becton Dickinson). The gating strategy is shown in Fig. 3a.

## Selective T-cell stimulation and therapeutic blockade

Cell suspensions ($n = 10$) were transferred into a 96-well flat-bottom plate with RPMI-1640 medium (Gibco™ RPMI 1640 Medium, Thermo Fisher Scientific) and cultured at a concentration of $0.3 \times 10^6/\mu l$ in the presence/absence of therapeutic mAbs. Five study arms were created. In the first study arm, non-stimulated (NS) cell suspensions were incubated for 24 h. In the cell suspensions of the second study arm, human T cells (CD4 + and CD8 +) isolated from tumor
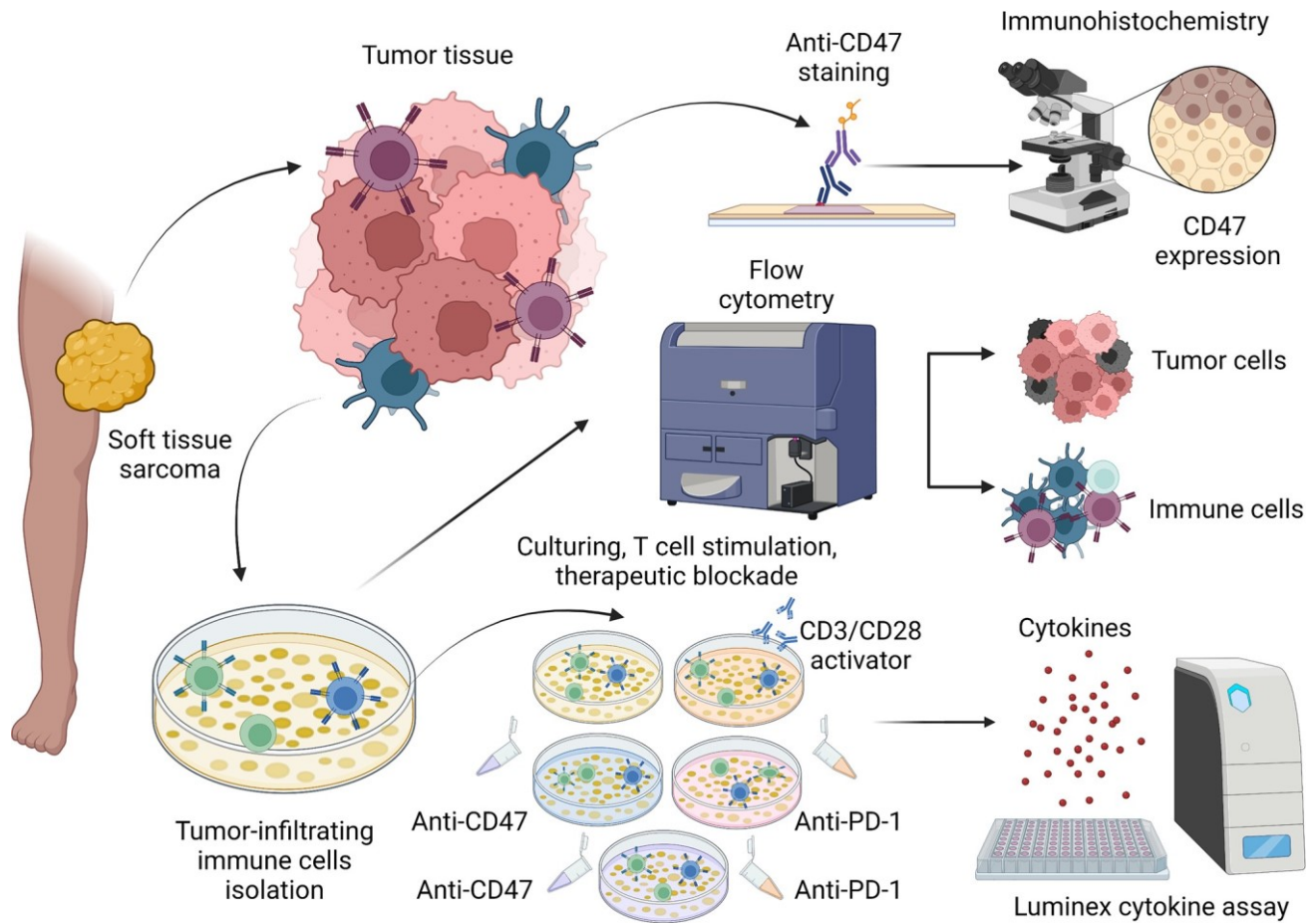
**Fig. 1** The study design. The cohort of 66 patients with STSs was first retrospectively screened for the expression CD47 in the tumor tissue by immunohistochemical analysis. Both tumor cells and immune cells were then analyzed by flow cytometry to allow the quantification of these populations. Five prospective study arms were created to investigate the immune cell activation in cell suspensions obtained from surgical resections. In the first study arm, NS cell suspensions were incubated for 24 h. In the second study arm, human T cells (CD4$^+$ and CD8$^+$) were activated by superparamagnetic polymer beads (CD3/CD28 activator). In the third study arm, cell sus-

pensions were prepared according to the procedure of arm two and incubated with an anti-PD-1 mAb. In the fourth study arm, cell suspensions were prepared according to the procedure of arm two and incubated with an anti-CD47 mAb. The fifth study arm included cell suspensions prepared according to the procedures of arms three and four (anti-PD-1 + anti-CD47 blockade). Cytokine secretion was analyzed using a multiplex Luminex cytokine bead-based immunoassay. This figure was created with BioRender.com (Agreement No. ZI23V8U8GU). *mAb* monoclonal antibody, *NS* non-stimulated, *STS* soft tissue sarcoma

samples were activated by superparamagnetic polymer beads with an optimized mixture of mAbs against CD3 and CD28 surface molecules (Dynabeads Human T-Activator CD3/CD28, Gibco, Thermo Fisher Scientific). The assay was performed according to the manufacturer's protocol. CD3 + T cells were stimulated with anti-CD3/CD28 beads for 24 h. In the third study arm, cell suspensions were prepared as described for arm two and incubated with anti-PD-1 mAb (Nivolumab, Selleckchem, Germany) at a final concentration of 2 µg/ml (Yao et al. 2021). In the fourth study arm, cell suspensions were prepared as described for arm two and incubated with anti-CD47 mAb (clone MIAP410, InVivoMab, BioXCell, Lebanon NH) at a final concentration of 20 µg/ml (Strizova et al. 2020). The fifth study arm included

cell suspensions prepared according to the above-described procedures for arms three and four (anti-PD-1 + anti-CD47 blockade).

## Luminex cytokine assay

Samples were prepared according to the manufacturer's recommendations, and supernatants from 50 cell suspensions (10 patients, 5 study arms) were analyzed using a multiplex Luminex cytokine bead-based immunoassay (R&D Systems). The Milliplex® MAP Human Cytokine/Chemokine Bead Panel (Sigma Aldrich) was used for the analysis of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and
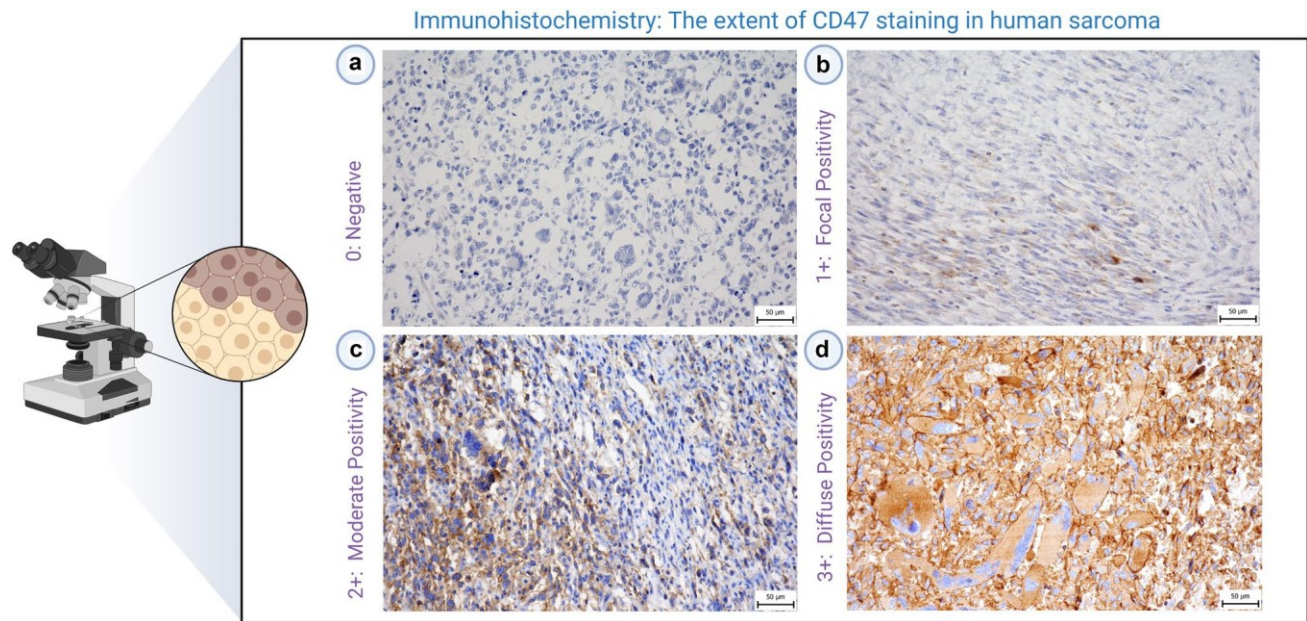
**Fig. 2** Immunohistochemistry (IHC) of CD47 expression in human soft tissue sarcomas. Representative images show negative CD47 staining in (**a**) pleiomorphic rhabdomyosarcoma (40x), focal positivity in (**b**) spindle cell rhabdomyosarcoma (40x), moderate positivity in pleiomorphic liposarcoma (40x) and diffuse positivity in d) undifferentiated pleiomorphic sarcoma (40x). This figure was created with BioRender.com (Agreement No. WG247EVV8B)

interleukin (IL)-2 cytokine levels. The procedure followed the manufacturer´s recommendations.

## Data analysis

Flow cytometry data were analyzed using FlowJo software (version 10.6.1). Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Statistical significance among three or more groups was measured using nonparametric one-way ANOVA with Dunn's multiple comparison test. Statistical significance between two groups of differentially treated samples was analyzed using Wilcoxon's matched-pair signed-rank tests. Statistical significance was tested at the $\alpha = 0.05$ level.

## Results

### The "don't eat me" signal is expressed in most soft tissue sarcomas

Previous studies have shown that in chondromas and angiosarcomas, up to 80% of cells express the CD47 molecule (Dancsok et al. 2020; Jiang et al. 2021; Zhang et al. 2020). Because the current study evaluating potential additive effects of anti-CD47 and immune checkpoint inhibitor immunotherapies included diverse histologic STS subtypes, we first investigated whether CD47 is expressed consistently within the study cohort. A scale ranging from 0 to 3 was used to quantify the extent of CD47 expression in the TME.

In our study, 86.9% of patients with STSs showed expression of CD47 molecule in the TME. In accordance with previously published studies, the highest expression of CD47 was observed in patients with angiosarcoma and chondrosarcoma. By contrast, leiomyosarcoma and rhabdomyosarcoma belonged to tumors with no CD47 expression in the TME. Of note, in our study cohort, only a limited number of angiosarcomas, chondrosarcomas, leiomyosarcomas, and rhabdomyosarcomas were present. The most frequent histological subtypes, liposarcomas and undifferentiated pleiomorphic sarcomas, displayed in most cases high staining of the total tumor area.

### Cytotoxic CD8^{+} T cells are the second most prevalent leukocyte subset in the sarcoma TME

While the high levels of CD47 detected in human STSs suggest that an anti-CD47 therapy targeting macrophages could represent a particularly effective treatment option, we hypothesized that we could further improve anti-tumor responses by stimulating other leukocytes in the TME.

Macrophages have already been shown to dominate the TMEs of sarcomas (Dancsok et al. 2020), and our flow cytometric analysis revealed that 40% of CD45^{+} cells are CD4^{+} and CD8^{+} T cells (Fig. 3b). This indicates that, after
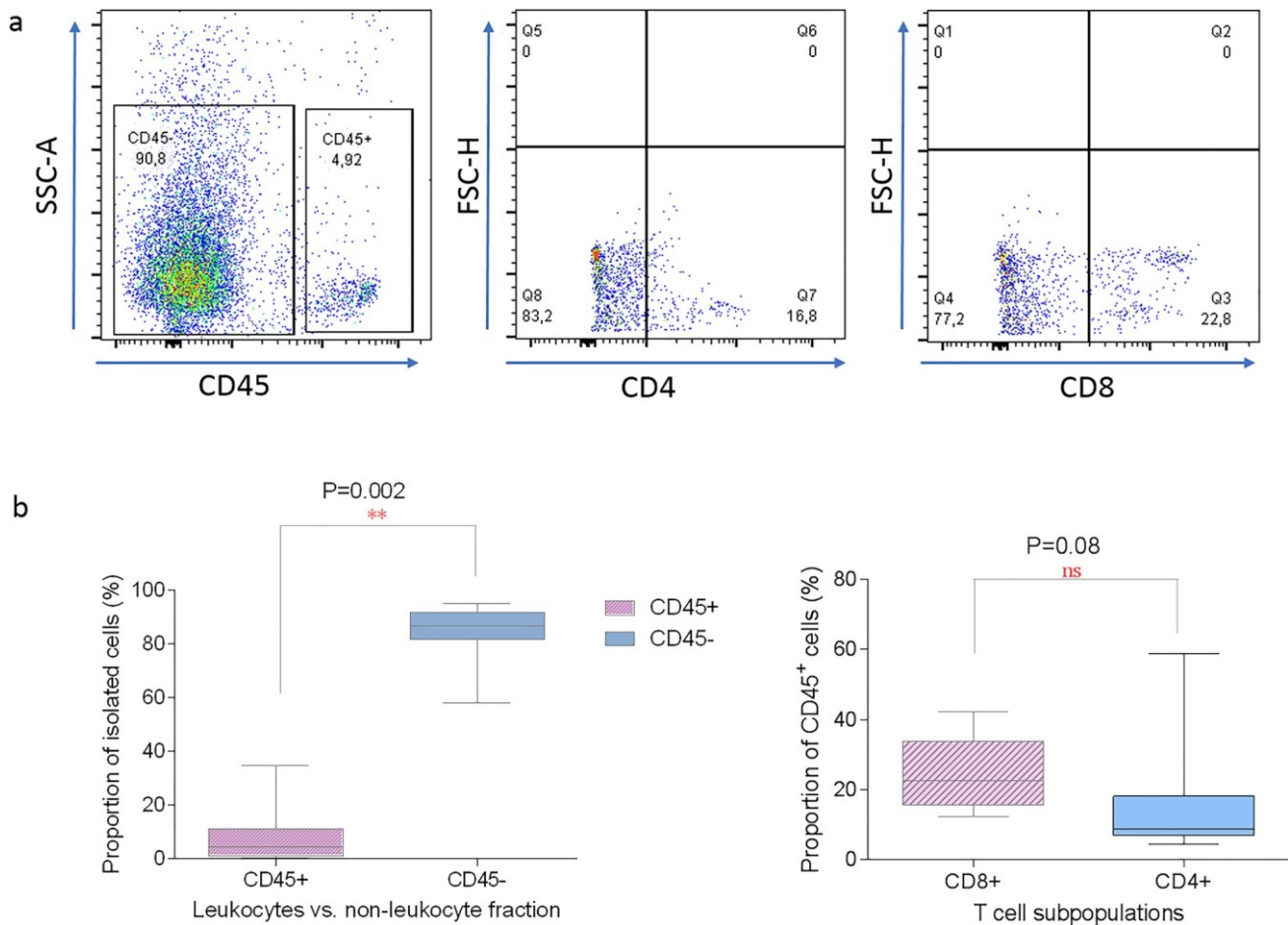
**Fig. 3** Gating strategy and the proportions of tumor-infiltrating cells. **a** The gating strategy for the analysis of flow cytometry data. **b** The analyzed cells were gated as in (**a**), and the proportions of leukocytes vs non-leukocytes (left graph) and CD4$^+$ vs CD8$^+$ T cells (right graph) in the tumor tissues of patients with STSs were determined by flow cytometry. The acquired data were analyzed using FlowJo Software (Tree Star, Ashland, OR). STS, soft tissue sarcoma

macrophages, T cells are the most prevalent leukocyte subset in human STSs. Furthermore, the proportions of cytotoxic CD8$^+$ T cells in TMEs were higher than those of CD4$^+$ helper T cells.

Collectively, these data suggest that a combinatorial therapy targeting both macrophages and CD8$^+$ T cells may have the potential to activate the vast majority of TILs, producing an even stronger anti-tumor response.

## Anti-CD47 therapy promotes strong immune cell activation in TMEs of soft tissue sarcomas

Anti-PD-1 therapy has already been shown to activate CD8$^+$ cytotoxic T cells (Verma et al. 2019). Thus, we aimed to determine whether combining anti-CD47 with anti-PD-1 therapy could activate TILs more effectively in vitro and promote the generation of an anti-tumor microenvironment characterized by increased secretion of specific pro-inflammatory cytokines which have been

shown to support tumor suppression (Berraondo et al. 2019).

Single-cell suspensions from tumor samples were split and exposed to five different conditions, including CD3/CD28 stimulation combined with anti-PD-1 therapy, anti-CD47 therapy, and most importantly, anti-PD-1 and anti-CD47 therapy simultaneously. Using a Luminex assay, concentrations of the pro-inflammatory cytokines IL-2, IFN-γ, and TNF-α were assessed, and these concentrations were used to infer the extent of TIIC activation and potential anti-tumor effect.

In NS cell suspensions, we did not detect any IL-2 or IFN-γ secretion and only minimal TNF-α production after 24 h of incubation. T-cell stimulation with anti-CD3/CD28 Dynabeads caused a modest increase in IFN-γ and TNF-α levels, but IL-2 production was still low (Fig. 4a).

The addition of anti-PD-1 to CD3/CD28 stimulation drastically increased the production of all cytokines analyzed,
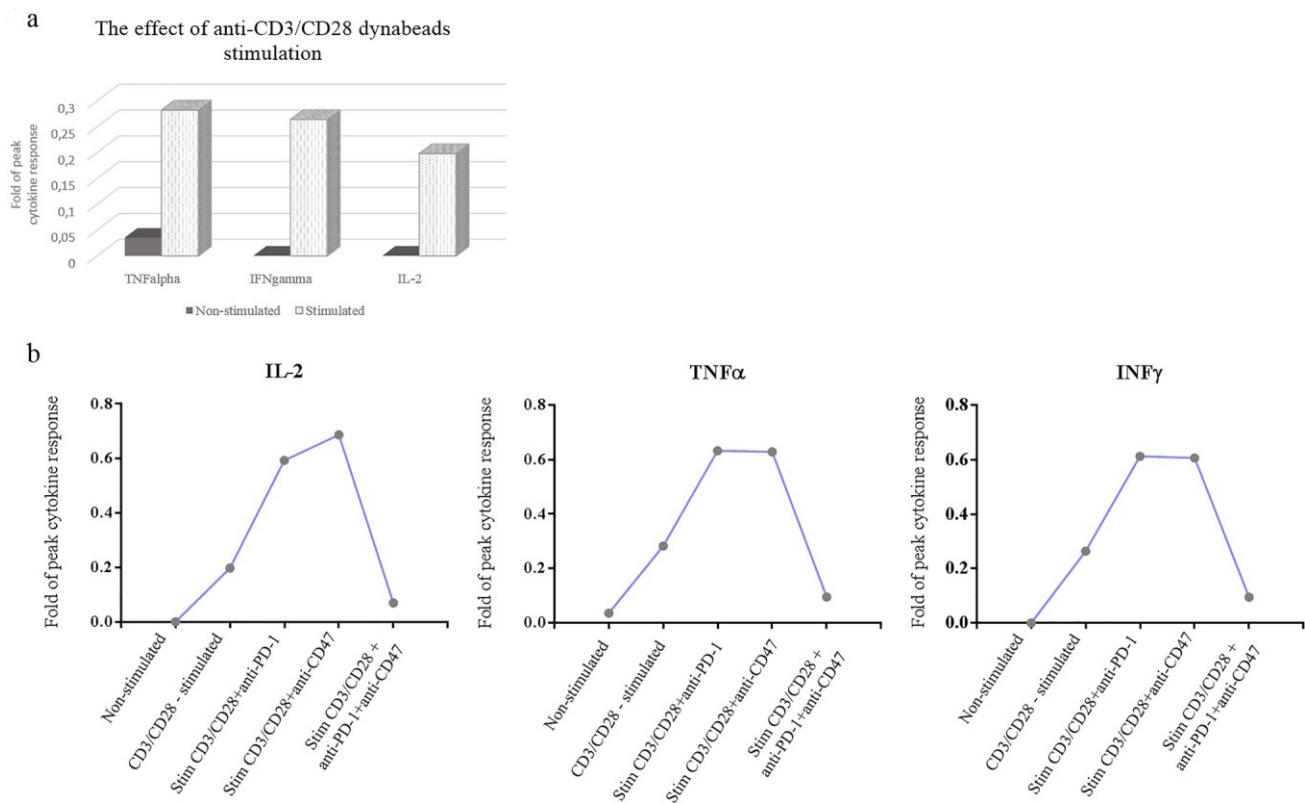
**Fig. 4** Cytokine responses in the study cohort. **a** Comparison of non-stimulated and anti-CD3/CD28-stimulated cells. Cytokine responses are displayed in a bar graph. **b** The effect of anti-CD47 and anti-PD-1 co-administration on immune cell activation in the TME of STSs in all patients. The concentrations of the pro-inflammatory cytokines IL-2, IFN-γ, and TNF-α were assessed to infer the extent of TIIC activation. Both anti-PD-1 and anti-CD47 therapies drastically increase the production of the pro-inflammatory cytokines IL-2, IFN-γ, and TNF-α in vitro. The co-administration of both agents causes a decrease in the pro-inflammatory immune response. *IFN* interferon, *IL* interleukin, *STS* soft tissue sarcoma, *TME* tumor microenvironment, *TNF* tumor necrosis factor

suggesting strong immune activation and the generation of a tumor-restrictive microenvironment.

This increase in pro-inflammatory cytokine secretion was even more pronounced after anti-CD47 therapy (Fig. 4b). However, combining anti-PD-1 and anti-CD47 treatment did not further increase cytokine secretion, but caused a decrease in cytokine levels which were more similar to those following CD3/CD28 stimulation alone. Thus, our findings suggest that anti-PD-1 and anti-CD47 therapies do not enhance each other and that the combinatorial application of anti-PD-1 and anti-CD47 agents in vivo may limit rather than potentiate their clinical efficacy.

## Leiomyosarcoma and undifferentiated pleomorphic sarcoma achieve peak cytokine responses after single-agent administration but do not respond to combinatorial treatment

Soft tissue sarcomas exhibit heterogeneity in their clinical behavior even within the same histological subtype, which complicates treatment and patient care (Skubitz et al. 2008). This is down to a variety of factors, such as different immune compositions or pre-existing comorbidities (Du et al. 2020; Skubitz et al. 2008).

Our study was initially designed to include histologically heterogeneous STSs, pooling all samples to examine whether a combinatorial therapy may represent a broad treatment option. Although the administration of both therapies appears to be ineffective when looking at the cohort, a combinatorial therapy targeting anti-CD47 and anti-PD-1 might still be effective against a particular STS subtype or in a particular set of patients. To explore this possibility, we attempted to re-evaluate trends in therapy responses, both on an individual level and by trying to group patients according to sarcoma type, age, and sex.

On an individual level, the most profound response to anti-PD-1 antibodies was observed in a high-grade leiomyosarcoma (Fig. 5a). In this case, the TNF-α production of anti-CD3/CD28-stimulated cells was 3.8 pg/ml but increased to 6019 pg/ml after 24-h incubation with anti-PD-1 antibodies.
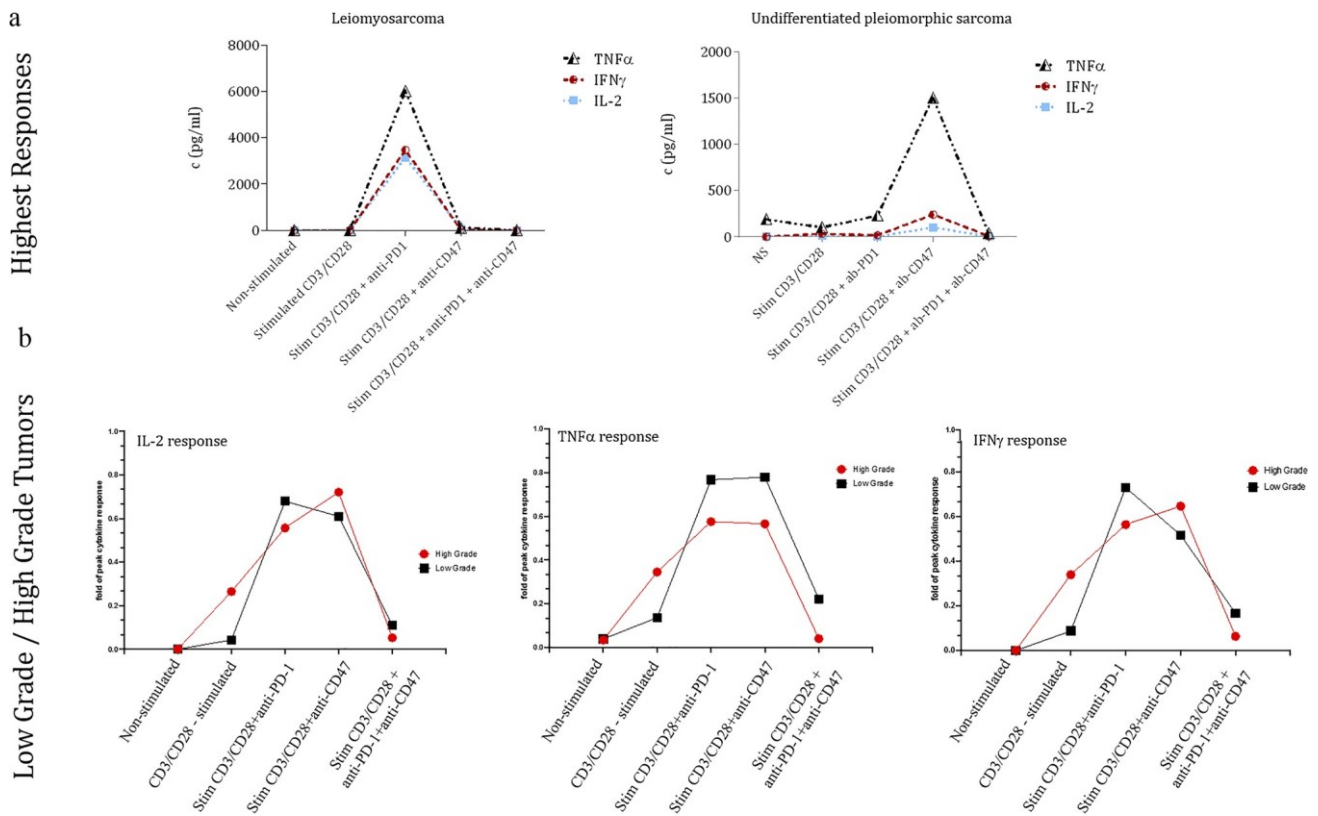
**Fig. 5** Pro-inflammatory responses in selected subgroups of patients. **a** The highest individual cytokine response to anti-PD-1 therapy was observed in leiomyosarcoma (left graph), and the highest individual cytokine response to anti-CD47 therapy was observed in undifferentiated pleomorphic sarcoma (right graph). The levels of IL-2, TNF-α, and IFN-γ are presented in pg/ml. **b** The correlation of pro-inflammatory cytokine responses between low-grade and high-grade STSs. The cytokine responses are normalized and presented as a fold of peak cytokine responses. *IFN* interferon, *IL* interleukin, *STS* soft tissue sarcoma, *TNF* tumor necrosis factor

The most profound response to anti-CD47 therapy was observed in an undifferentiated pleomorphic sarcoma, where the TNF-α production of anti-CD3/CD28-stimulated cells increased from 101 to 1503 pg/ml after the addition of anti-CD47 antibodies (Fig. 5a).

However, regardless of the individual therapy achieving peak cytokine responses, all patient samples treated with a combination of both anti-PD-1 and anti-CD47 antibodies showed a dramatic reduction in cytokine secretion.

This effect was also seen when grouping patients according to sarcoma grade (high vs low), sarcoma type (pleiomorphic vs liposarcoma), age (below 65 vs above 75), and sex (men vs women; Figs. 5b, 6 and 7). In all cases, the peak cytokine response was achieved by the administration of anti-PD-1 or anti-CD47 antibodies in combination with CD3/CD28 stimulation. When both treatments were administered simultaneously, cytokine secretion levels dropped below the levels seen with CD3/CD28 stimulation alone, indicating a profound inhibition of the inflammatory response.

# Discussion

To date, most patients with STSs die of metastatic disease within a few years after the initial diagnosis (Komdeur et al. 2002; Lochner et al. 2020). While the incorporation of novel surgical techniques has led to major improvements in the local control of localized STSs, treatment options for metastatic STSs are limited (Bonvalot et al. 2010; Gronchi et al. 2013; Lochner et al. 2020; Ozaniak et al. 2020). Systemic chemotherapeutic agents induce a response in only 15–35% of the patients, irrespective of the histological subtype (Ozaniak et al. 2021). Both neoadjuvant and adjuvant radiotherapies reduce local recurrence rates but are also associated with considerable toxicity, especially in retroperitoneal STSs (Chouliaras et al. 2019; Ozaniak et al. 2021). Histotype-tailored approaches are currently widely discussed in STS, especially metastatic STS, because specific cytotoxic drugs were shown to be quite effective in distinct STS histotypes (Gronchi et al. 2012; Higham et al. 2017). On the other hand, the phase III clinical trial by Gronchi et al. demonstrated significantly worse disease-free survival and overall survival in the histotype-tailored group compared to the standard
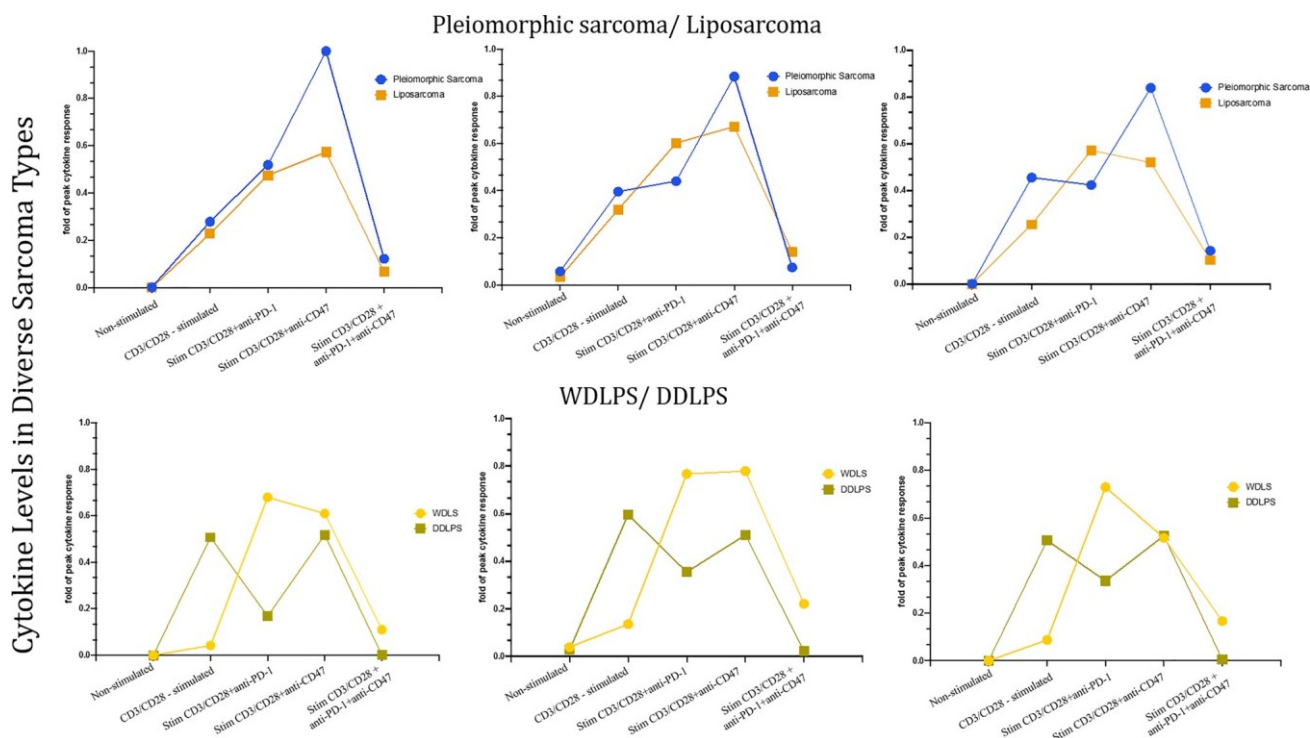
**Fig. 6** Pro-inflammatory cytokine concentrations in selected histological subtypes of STSs. The correlations of pro-inflammatory cytokine responses between pleiomorphic sarcomas and liposarcomas (upper row) and between well-differentiated liposarcoma and dedifferenti-ated liposarcoma (lower row) are shown. The cytokine responses are normalized and presented as a fold of peak cytokine responses. *STS* soft tissue sarcoma

chemotherapy group (Gronchi et al. 2017). However, the differential chemosensitivity across diverse histological subtypes and grades of STSs suggests the need of personalized treatment of these rare disease entities (Bleloch et al. 2017; In et al. 2017). Similarly, a broad spectrum of radio-sensitivities exists across STS cell lines which is reflected in subtype-specific radiation responses (Haas et al. 2021). As for immunotherapy with immune checkpoint inhibitors, even with the so far disappointing response rates in STSs, some histological subtypes were still found to benefit more than others (Roulleaux Dugage et al. 2021).

In the hope of reducing the risk of both local recurrence and distant metastases in STSs patients, inventing novel therapy approaches and searching for novel neoadjuvant/adjuvant treatment options is crucial (Ozaniak et al. 2021).

Immunotherapy represents a breakthrough in the treatment of metastatic diseases (Bang and Schoenfeld 2019; Strizova et al. 2021). Immune checkpoint inhibitors have become the first-line treatment for various solid tumors, such as metastatic renal cell carcinoma or metastatic non-small cell lung carcinoma (Tung and Sahu 2021; Xiao et al. 2021). In STSs, however, immune checkpoint inhibitors did not show a significant effect on tumor growth (Saerens et al. 2021). One of the main reasons might be the relatively mild infiltration of STSs with TILs (Raj et al. 2018). On the other hand, the TME of STSs is highly infiltrated with macrophages which opens new avenues for macrophage-targeted immunotherapies (Dancsok et al. 2020).

In the current study, we evaluated the potential additive effects of combining anti-PD-1 and anti-CD47 treatment to activate TILs in human STSs. Studies on the combined therapy using an immune checkpoint inhibitors (anti-CTLA-4) with anti-CD47 have previously been carried out in a mouse model of melanoma (Schwartz et al. 2019). To our knowledge, our study is the first to evaluate the potential additive effect of anti-PD-1 and anti-CD47 therapy in human STSs.

We observed that, after macrophages, the next most common type of CD45^{+} leukocytes in human STSs are CD3^{+} T cells, with the cytotoxic CD8^{+} T-cell subset being more prevalent than the CD4^{+} subset. The high proportion of cytotoxic T cells suggests that a therapy designed to efficiently target T cells in the TME may offer great potential to prevent disease progression.

Our findings show that both anti-PD-1 and anti-CD47 therapies drastically increase the production of the pro-inflammatory cytokines IL-2, IFN-$\gamma$, and TNF-$\alpha$ in vitro. However, our hypothesis that co-administration of anti-CD47 and anti-PD-1 would be more effective than either treatment alone was not confirmed, and even with diverse treatment responses in our histologically heterogeneous
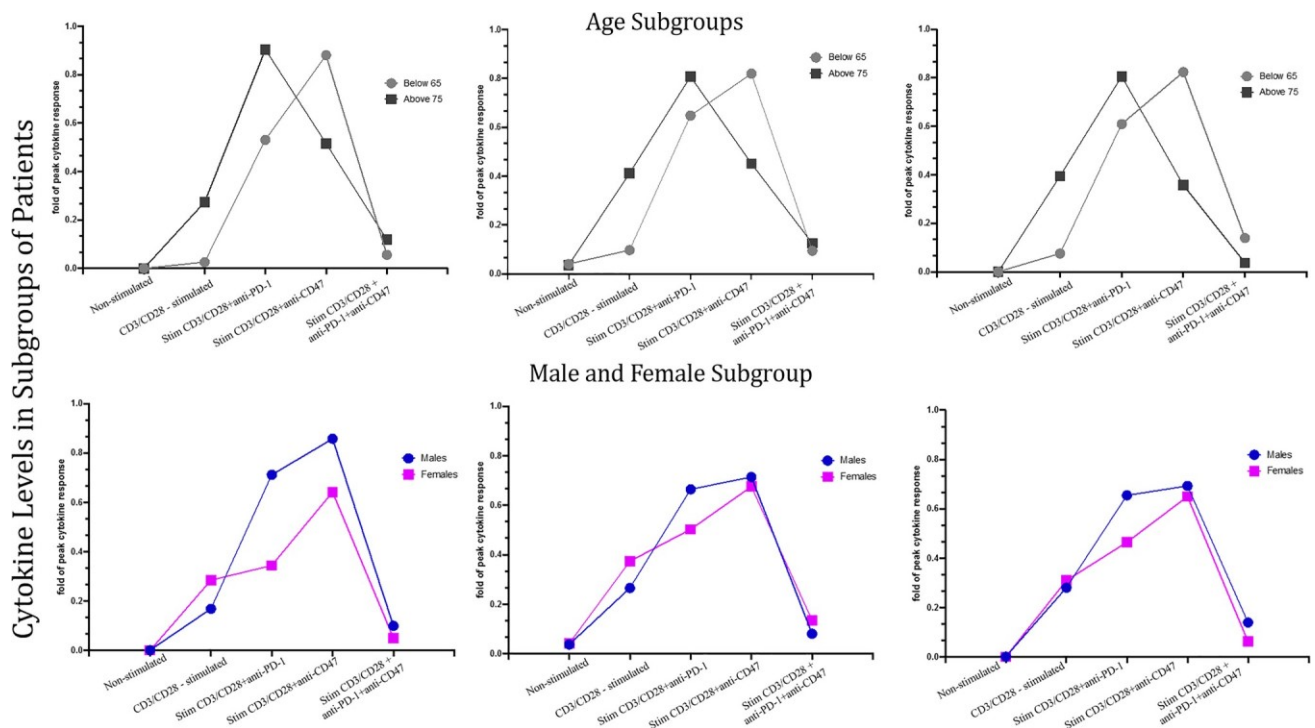
**Fig. 7** Immune cell activation after anti-PD-1/anti-CD47 treatment in times of STSs in various age and sex subgroups. The correlation of pro-inflammatory cytokine responses between the youngest patients and the oldest patients in the study cohort (upper row). Immune responses to anti-PD-1 and anti-CD47, as well as responses to both agents simultaneously, in women and men (lower row). The cytokine responses are normalized and presented as a fold of peak cytokine responses. *STS* soft tissue sarcoma, *TME* tumor microenvironment

cohort, uniform and consistent tendencies with a major decrease in T-cell responses were observed in all patients after combined administration of anti-CD47 and anti-PD-1 agents.

Recent studies have shown that the activation of anti-tumor T cells by anti-PD-1 is not direct but rather involves a crosstalk between T cells and dendritic cells (Garris et al. 2018). This mechanism is also strongly mediated by the cytokines IFN-γ and IL-12 (Garris et al. 2018). With dual administration of anti-CD47 and anti-PD-1 agents, we hypothesize there might be competing cascades of reactions that ultimately decrease the efficacy of both treatments. First, anti-CD47 therapy enhances tumor cell phagocytosis by both M1 and M2 macrophage subtypes (Zhang et al. 2016). This therapy-induced activation of macrophages may also promote cytokine release by tumor-associated macrophages which are the most prevalent macrophage subtype in the TME (Boutilier and Elsawa 2021). Since macrophage polarization can be altered based on the integration of multiple signals from other cell types in the TME, this mechanism may also significantly change the spectrum of TIICs (Boutilier and Elsawa 2021; Chen et al. 2019). Second, the cytokines produced by tumor-associated macrophages include anti-inflammatory cytokines, transforming growth factor β and IL-10 (Chen et al. 2019) These cytokines are

known to cause CD8 T-cell inhibition, as well as regulatory T-cell activation (Thepmalee et al. 2018). Anti-PD-1 therapy has also been demonstrated to induce IL-2 secretion by T-cells which may also contribute to the regulatory T cell expansion within the TME (Chiu et al. 2022; Stecher et al. 2017). The suppressive functions of regulatory T cells further inhibit dendritic cells in presenting tumor antigens to activate CD4$^+$ and CD8$^+$ T-cells (Thepmalee et al. 2018). The missing crosstalk between TILs and tumor-infiltrating dendritic cells may further impair the efficacy of anti-PD-1 immunotherapy. Therefore, while anti-PD-1 can enhance T-cell function, the addiction of an anti-CD47 therapy might induce phenotypic changes in tumor-associated macrophages and dendritic cells, which in turn could decrease the effectiveness of anti-PD-1 therapy. Another possible mechanism involved could be the anti-CD47-induced lymphodepletion which may occur in cancer types with high CD47 expression on TILs (Strizova et al. 2020).

Unfortunately, we still have a limited understanding of how anti-CD47 and anti-PD-1 treatments engage complex TMEs and which mechanisms define the treatment success.

Of note, the highest concentration of TNF-α was observed in high-grade leiomyosarcoma after the in vitro administration of anti-PD-1. Leiomyosarcoma is characterized by both mild infiltration with macrophages and infrequent expression

of CD47; thus, patients with leiomyosarcoma might benefit from PD-1 therapy rather than from anti-CD47 therapy (Lazar et al. 2017; Ganjoo et al. 2011; Lee et al. 2008). Our data support the findings of these studies; however, the study by Edris et al. reported a highly effective anti-CD47 treatment in a murine model of leiomyosarcoma (Edris et al. 2012). Thus, more studies in leiomyosarcoma are needed.

In our study, the most profound response to anti-CD47 therapy was observed in an undifferentiated pleomorphic sarcoma which also belonged to the histological subtypes with the highest expression of CD47. Tumor-associated macrophages frequently infiltrate pleomorphic sarcomas, such as undifferentiated pleomorphic sarcoma and dedifferentiated sarcoma (Dancsok et al. 2020). Therefore, anti-CD47 agents in the treatment of pleomorphic sarcomas might have a dual effect by potentiating macrophage-mediated phagocytosis and promoting T-cell cytotoxicity (Chen et al. 2021; McCracken et al. 2015). On the other hand, CD47 was widely expressed in other STS subtypes, such as high-grade myxofibrosarcomas or angiosarcomas, where the efficacy of anti-CD47 therapy was rather limited. We have previously shown that anti-CD47 mAbs may also impact tumor-infiltrating lymphocytes and, thus, be detrimental to the efficacy of anti-CD47 immunotherapy (Strizova et al. 2020). Whether this mechanism also comes into play in myxofibrosarcomas or angiosarcomas is currently unknown.

Our data suggest that combinatorial strategies based on immune CPI and anti-CD47 blockade may not provide the desired outcome. Both therapies, either anti-PD-1 or anti-CD47 alone, could, however, bear a therapeutic potential in selected tumors after close evaluation of the CD47 and PD-1 expression status, as well as the extent of TIL/macrophage infiltration.

## Declarations

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# 5 ZÁVĚR

Chirurgická léčba je základním kamenem léčby lokalizovaných STS. Naopak léčba metastatických STS zůstává velkou výzvou vzhledem k limitované chemosenzitivitě těchto nádorů. Imunoterapie je slibnou léčebnou modalitou, která se dostala v posledních letech do první linie léčby celé řady metastatických nádorových onemocnění, avšak v léčbě STS zatím nemá své místo. Jedním z důvodů může být doposud velmi omezené zmapování nádorového mikroprostředí STS a současně extrémní histologická heterogenita těchto nádorů, která brání koncepci racionálních klinických studií s jednotlivými imunoterapeutiky. Závěry vyplývající z našeho výzkumu zdůrazňují, že centralizace pacientů je jedním z nejdůležitějších aspektů ovlivňujících přežívání pacientů. Současně jsme naší prací poukázali na nutnost pohlížení na konkrétní histologické typy STS, a nikoliv na celou heterogenní kohortu bez ohledu na histologii. V neposlední řadě naše data ukázala, že ne všechna imunoterapeutika mají synergický efekt a je nutné dále in vitro testovat různé kombinace terapií, abychom poskytli pacientům s metastatickým STS naději na nové možnosti kombinovaných systémových přístupů.

# 6    VLASTNÍ PUBLIKACE

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