MASARYKOVA UNIVERZITA
LÉKAŘSKÁ FAKULTA
KLINIKA DĚTSKÉ ONKOLOGIE

Diagnostické a léčebné pokroky v dětské onkologii

Habilitační práce v oboru onkologie

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Komentář

ÚVOD: Po relativně stabilním období přichází v poslední dvou desetiletích rychlý vývoj diagnostických a molekulárních postupů v dětské onkologii. Zvláště je tento vývoj patrný jednak na implementaci molekulárně genetických metod do klinické praxe, a také na dostupnosti nových léčiv na jiných principech než dosud standardní chemoterapie.

CÍLE: Cílem práce je podat přehled ve vývoji těchto metod za poslední dvě desetiletí a popsat na komentovaném souboru prací jejich přínos pro klinickou praxi.

SOUBOR PACIENTŮ A METODY: Inovativní léčebné postupy, diagnostika a terapie invazivních mykóz a léčba sarkomů měkkých tkání jsou tři oblasti, které byly extenzivně zkoumány v publikovaných pracích. Na Klinice dětské onkologie Lékařské fakulty Masarykovy univerzity a Fakultní nemocnice Brno bylo v letech 2000–2020 více projektů, které se zabývaly uvedenými tématy. Pokrývají spektrum metronomické antiangiogenní terapie, použití tyrozinkinázových inhibitorů v nových indikacích, somatobuněčnou terapii autologními dendritickými buňkami, diagnostiky a terapie invazivních mykóz a diagnostické a terapeutické postupy u sarkomů měkkých tkání u dětí.

VÝSLEDKY: Mnohá léčiva v kategorii inovativních postupů byla použita zcela poprvé, resp. s nejlepším publikovaným výsledkem, jako např. vakcinace proti HPV z indikace rekurentní laryngeální papilomatózy nebo léčba infantilní myofibromatózy u pacienta s germinální mutací PDGFRB genu. Zavedená antiangiogenní metronomická terapie má přínos pro pacienty s vysoce rizikovými nádory, personalizace léčby vedla i k neočekávaným kurativním výsledkům, které nebylo možné očekávat při použití konvenční léčby. Participace na projektech diagnostiky a léčby invazivních mykóz vedla ke kvalitní péči o tyto pacienty, jak je dokumentováno i v publikovaných pracích, i za použití inovativních léčebných postupů mimo klinická hodnocení. Rozvoj somatobuněčné terapie je spojen s novými technologickými postupy výroby dendritické vakcíny a se standardizací průběhu klinických hodnocení tohoto typu, ve formě protokolu KDO DC1311, dosud jediného provedeného u dětí do 18 let v České republice. Nové poznatky, které jsou tímto klinickým hodnocením získány, jsou inovativní a přispívají k dalšímu rozvoji této slibné léčebné metody. Participace na projektech mezinárodních akademických klinických studií diagnostiky a léčby sarkomů měkkých tkání vedly k velmi cenným výsledkům ve formě standardizace doporučených léčebných postupů u těchto diagnóz, k prokázání účinnosti udržovací léčby u rabdomyosarkomu jako první takový

důkaz u solidních tumorů, k nové stratifikaci léčby u alveolárních rabdomyosarkomů s postižením lymfatických uzlin a k dosud nejlepším léčebným výsledkům u extrakraniálních maligních rabdoidních tumorů.

ZÁVĚR: V publikovaných pracích jsou shrnuty recentní výsledky rozvoje diagnostických metod a léčby u malignit dětského věku se zaměřením na vysoce rizikové pacienty bez možnosti konvenční léčby i standardizace léčebných postupů u invazivních mykóz a sarkomů měkkých tkání při participaci v mezinárodních multicentrických prospektivních akademických klinických hodnoceních.

Commentary

INTRODUCTION: A quite standard treatments known two decades ago were followed by rapid progress in molecular diagnostics and treatment possibilities in paediatric oncology. New moleculal biology methods and new treatments based mainly on theranostics principles are used in daily practice.

OBJECTIVES: The aim of this work is to review how far the paediatric oncology was updated during last two decades and to show this framework in commentary of published papers.

PATIENTS AND METHODS: Innovative therapies, diagnostics and treatment of invasive fungal infections and treatment of soft tissue sarcomas are areas covered by published articles. There are a lot of projects on Paediatric Oncology Department of School of Medicine Masaryk University and University Hospital Brno during period 2000-2020, which cover topics as metronomic antiangiogenic therapy, tyrosinkinase inhibitors in new therapeutic indications, cell therapies with autologous dendritic cells, new therapeutic strategies of invasive fungal infections and diagnostics and therapeutic approaches in soft tissue sarcomas in children.

RESULTS: Many of innovative therapies were used for first time ever or with the best results published. This is the case of human papillomavirus vaccination against recurrent laryngeal papillomatosis or successful management of infantile myofibromatosis in patients with germline mutation of PDGFRB gene with tyrosinkinase inhibitor. Introduced antiangiogenic metronomic therapy is successfully used in patients with high risk tumours. Personalised therapies were administered to patients with sometimes surprising results even curative which were not anticipated with conventional approach. Setting quality of diagnostic and therapeutic tools in patients with invasive fungal infections was substantial for good treatment results and was accompanied by novel therapies used off-label and off-clinical trial as published. New cell therapy with dendritic cells was introduced and this accelerated rapid biotechnology development and proper academic trial administration in era of new and tight law regulations. KDO DC 1311 trial is the only open for children in the Czech Republic, this trial is still active. It generated some new knowledges on monocyte response to anticancer therapies or host immune response against cancer which are used in clinical practice and support further development of such promising method. Multicentric prospective international clinical trials

on diagnostics and treatment of soft tissue sarcomas were essential for generating of standardized therapy across Europe, new treatments such as maintenance chemotherapy in rhabdomyosarcoma which is the first robust evidence about its efficacy in solid tumours, new treatment stratification in alveolar rhabdomyosarcomas with regional nodal involvement and improvement treatment of highly aggressive malignant rhabdoid tumours.

CONCLUSIONS: Recent progress in diagnostics and therapies in childhood malignancies are described in published articles with focus to high risk patients without possibility of conventional treatment and to standardization of treatment of invasive fungal diseases and soft tissue sarcomas while participating in international multicentric prospective academic driven clinical trials.

1 Inovativní léčebné postupy u vysoce rizikových malignit dětského věku

1.1 Úvod

Léčebné výsledky jsou u malignit dětského věku jako celku velmi dobré. Kurativní postupy vedou k trvalému vyléčení u více než 80 % dětských pacientů, byť za cenu jisté míry pozdních následků, které si s sebou pacienti do života odnáší. Existují ovšem velké rozdíly v přežití malignity mezi jednotlivými diagnostickými skupinami, které při celkovém hodnocení malignit dětského věku jako celku nejsou, z důvodu rozdílné incidence, na první pohled patrné: ve skupině akutních lymfoblastických leukémií, Wilmsových nádorů, germinálních nádorů, nízce rizikových sarkomů se úspěšnost léčby blíží 90 %. Naopak gliomy mozkového kmene, glioblastomy, metastatické sarkomy skeletu a měkkých tkání nebo relabované Burkittovy lymfomy jsou léčitelné v malém procentu případů s mediánem přežití do šesti měsíců. Právě tato skupina malignit je předmětem zájmu inovativních léčebných postupů. V posledních letech významným způsobem pokročilo poznání biologie nádorových onemocnění, které vedlo k identifikaci charakteristických znaků („hallmarks“) nádorů, poskytujících nádorové buňce a tkáni růstovou výhodu. Tyto aberace se v posledních letech snažíme léčebně ovlivňovat, a to tak, že pečlivě vyšetřeným pacientům je nabízena terapie na základě přítomnosti či nepřítomnosti určitých aberací či biomarkerů.

1.2 Personalizovaná onkologie

Základní premisou personalizované onkologie je, že každý nádor je biologicky unikátní a v čase se může i velmi významně měnit. Množství poznatků o biologii nádorů, jejich genetickém pozadí, imunologických aspektech jejich vzniku a možnostech terapie rychle narůstá. Je pravděpodobné, že tradiční velké, tisícihlavé randomizované prospektivní studie budou často obsoletní dříve, než budou samy dokončeny. Toto je v případě léčby sarkomů známo u mnoha „nadějných“ kinázových inhibitorů, jako např. recentně u olaratumabu. Klasická paradigmata klinického výzkumu v onkologii začínají respektovat realitu, dokumentovanou obrovskou genetickou a epigenetickou heterogenitou morfologicky podobně vypadajících procesů. Tyto

informace dříve nebyly k dispozici. Dnes reflektujeme individuální variabilitu hostitele, tedy nositele nádorového onemocnění, a tím potřebu indikovat pacientovi v podstatě „léčbu na míru“, než abychom se drželi překonaného přístupu „one size fits all“ a monoterapií. Jednoznačným důvodem je snaha podávat správné léky ve správných kombinacích, ve správný čas a správnému pacientovi. Tato strategie se musí opírat o komplexní biologickou analýzu nádorové tkáně, musí být doplněna o detailní informace o hostiteli, např. o genotypizaci a fenotypizaci enzymů, ale také o mikrobiomu pacienta, který se zdá být velmi důležitý např. pro odpověď pacientů na protinádorovou imunoterapii. Výsledky řady studií naznačují, že cílené – „targeted“ – terapie lépe fungují na časné fáze nádorového onemocnění, jak ukazuje příklad obrovské efektivity inhibitorů bcr/abl u nově diagnostikované chronické myeloidní leukémie. A tím je vysoká pravděpodobnost selhání identické terapie, pokud je u stejné nemoci podávána v pozdních fázích. I přes tuto zkušenost je v oblasti solidních nádorů dětí i dospělých cílená léčba pacientům často nabízena až v pozdních, pokročilých fázích onemocnění, a většinou je přínos takové terapie velmi problematický, ne-li vůbec žádný. Imunoterapie, na rozdíl od cílené terapie, může být efektivní i u některých pacientů s pokročilým onemocněním, tedy často s vyšší mutační náloží.

Další velmi problematickou oblastí je také dávkování a načasování podání cílených léků. V řadě případů se firmy stále drží konceptu maximálně tolerované dávky, i když např. dávka potřebná k zastavení fosforylace příslušné signální dráhy je mnohdy jen zlomkem dávky cytotoxické. Příliš vysoká dávka pak vede k významné toxicitě nových léků, zejména pokud jsou kombinovány se standardní chemoterapií. Nevhodné dávkování pak může vést k tomu, že potenciálně efektivní terapie se pak řadu let nepoužívá, a k renesanci dojde až po snížení podávaných dávek. Příkladem zde může být velmi komplikovaný příběh gemtuzumab-ozogamycinu, který se testoval již na přelomu tisíciletí, a po dlouhé přetržce se vrací na scénu jen ve významně redukovaných, a tedy lépe tolerovaných dávkách. Darwinistické vnímání procesů resistence je sice v medicíně teoreticky akceptováno, ale v reálné klinické onkologické praxi není reflektováno prakticky vůbec. Současná praxe podávání stejné terapie až do jasné klinické či radiologické progrese onemocnění bere onkologovi (a současně i pacientovi) výhodu zvažovat a realizovat léčbu alespoň jeden tah kupředu. Zajímavým konceptem by zde mohlo být například využití tzv. „liquid biopsies“, kdy je možno pomocí sekvenování nové generace detekovat cirkulující nádorovou DNA, a takto dříve detekovat objevení se nové, resistentní mutace či klonální evoluce – a tedy nástup resistence k podávané terapii. Identifikace klinicky relevantních informací pomocí sofistikovaných molekulárně genetických metod (např. NGS,

včetně „ultra deep“ sekvenování) z periferní krve či mozkomíšního moku může v některých případech nahradit rizikové, či třeba zcela nedostupné biopsie z nádorové tkáně a může pomoci ve sledování i klonální evoluce zpočátku minoritních klonů v průběhu času.

1.3 Klinická hodnocení nových léčiv v dětské onkologii

Filozofie přístupu k inovativním léčebným postupům jsou prakticky dvě. Na jedné straně klasický model monoterapie novým léčivem s vyhodnocením preklinických experimentů, poté klinických fází I–III s vyhodnocením toxicity, popisem farmakologických vlastností a léčebných odpovědí. Takto nastavené testování je vhodné pro onemocnění s vyšší incidencí nebo z velkého počtu center, kdy je možné během relativně krátké doby vyhodnotit na reprezentativním vzorku pacientů účinnost léčby monoterapií. V dětské onkologii je toto testování v prvních fázích doménou velkých nadnárodních kooperativních skupin center dětské onkologie, největší z nich jsou severoamerická Children´s Oncology Group nebo evropské ITCC. Zatímco v počátcích klinické onkologie bylo toto testování základem kurativních postupů s použitím cytostatické léčby, která v dětské onkologii zůstává nadále dominantní a vedla k průkopnickým pracím, na jejichž základě byla chemoterapie v klinické onkologii vybudována i pro malignity dospělého věku, v poslední dekádě je patrný posun opačným směrem, kdy zkušenosti u malignit dospělého věku jsou rychle přenášeny do onkologie dětské. Příkladem jsou rychle rostoucí zkušenosti s imunoterapií „check-point“ inhibitory, u nichž se velmi rychle rozšiřují indikace u nádorů typických pro dospělý věk, ale velmi pomalu se získávají zkušenosti u nádorů dětského věku. Jednou z mála výjimek je použití principu CAR-T buněčné terapie, která vznikla jako výsledek akademického výzkumu v Dětské nemocnici ve Filadelfii ve Spojených státech amerických. Metodologie populačního testování je postavena na klasické statistické analýze. Druhým přístupem je kombinované léčba, kdy je předmětem zkoumání účinnost dosavadních postupů s přidáním nového léčiva. V dětské onkologii probíhá v posledních letech testování velkého počtu tyrozinkinázových inhibitorů, které jsou přidány ke stávající chemoterapii. Tento postup vede zřídka k úspěchu, neboť je zatížen značnou toxicitou. To je z větší části dáno nastavením chemoterapeutických léčebných schémat, tedy podáváním maximálně tolerovaných dávek, kdy přidání dalšího léčiva, byť s odlišným mechanismem účinku, již naráží na toleranci organismu a není možné konkomitantně takovou léčbu kombinovat. Legitimní přístup je u vzácných onemocnění a kombinované terapie použití tzv. „N-of-1 trial“ metodologie analýzy, která počítá s historií

léčby pacienta jako s hodnocenou proměnnou, a je možné díky ní zjistit, zda má použití léčebné intervence přínos pro jednotlivého pacienta, na rozdíl od populačních analýz, které určují benefit pro skupinu pacientů.

1.4 Nové metody získávání dat v personalizované onkologii

Významným faktorem, který komplikuje další zlepšování léčebných výsledků v dětské onkologii, je orientace většiny klinických studií na samotné léčivo, především s cílem jeho registrace, což je pochopitelné u farmaceutických společností, ale méně to již reflektuje oprávněné zájmy konkrétního pacienta. Pacient je tak hledán pro potřeby studie, a daleko méně často míří studie či léčiva za pacientem. Právě zlepšení přežití, nejen léčebných odpovědí, bude vyžadovat mnohem větší dynamičnost a modulace léčby podle měnící se biologie nádoru, optimálně ještě před přirozeným nástupem resistence. To však bude samozřejmě vyžadovat také změny současného regulačního kontextu a charakteru klinických studií směrem k tzv. „N-of-1 trials“. Při této metodologii, na rozdíl od stávajícího populačního přístupu, je každý pacient sám sobě opakovaně v čase kontrolou, jako například při sledování krevního tlaku u individuálního pacienta („single patient trials“). Na stejném principu je založena i tvorba individuálního biologického profilu (pasu) u vrcholových sportovců. Populací je tedy jeden jediný pacient a vzorek pro analýzy se skládá z opakovaných měření efektu různé léčby u téhož pacienta. Jsou-li doba do progrese či přežití u pacienta na personalizované léčbě významně delší nebo srovnatelné a s lepší kvalitou života než při předchozím použití standardní (populační) léčby, pak to lze považovat za podporu takového přístupu. Je nutno zdůraznit, že zde není hlavním testovaným předmětem určitý lék, ale hlavním testovaným prvkem je právě přístup. Tedy to, zda komplexní molekulární charakterizace nádoru a jeho hostitele, a v případě potřeby i opakovaná, je tou správnou cestou. Tento přístup však zatím jen obtížně hledá uplatnění v rámci dosud velmi rigidně nastavených systémů regulací a úhrad léčivých přípravků, tak aby systém, který byl historicky nastaven s cílem ochraňovat pacienty, nebyl dnes spíše překážkou, bránící efektivní terapii respektující individualitu pacienta a jeho nemoci.

Další možností, jak zlepšit šanci pacientů dostat se ke správné léčbě, je větší využívání klinických a laboratorních dat generovaných v reálném klinickém provozu. V rámci klinických studií je léčeno méně než 5 % onkologicky nemocných, tedy je zde obrovská populace pacientů,

kteří do klinických studií zařazování nejsou. Obrovskou, a dosud jen málo využívanou příležitostí je využít různé, ale dobře a řádně vedené registry, které mohou poskytovat nesmírně cenné informace. Tyto registry je pak třeba možno použít jako kontrolní ramena pro „single arm studies“, testující nové postupy na malých a biologicky dobře definovaných skupinách pacientů, kde klasické randomizované studie nejsou reálné.

Zcela zásadní jsou v této souvislosti dostatečně spolehlivé biomarkery, které by měly zahrnovat optimálně multiomický přístup. Ani tehdy, kdy nenacházíme cílitelnou mutaci na úrovni DNA, to neznamená, že žádná genová aktivace přítomna není. Například gliomy jsou velmi náchylné k reaktivaci vývojových signálních drah i bez mutací na úrovni DNA. Stejně tak, imunitní únik nádoru („immune evasion“) a angiogenese nenastává na úrovni DNA, a tedy biomarkery opírající se výhradně o aberace na úrovni DNA, včetně stávajících komerčně dostupných panelů, zde mohou selhávat.

Je proto zapotřebí zvažovat inkorporaci biomarkerů na úrovních RNA, proteinů, jejich fosforylace, včetně stanovení mutační nálože a mutačního podpisu nádoru („mutational signature“), a to vše v kontextu velmi pečlivě zhodnoceného nádorového mikroprostředí a cirkulujících biomarkerů, jako např. T-regulačních lymfocytů.

Postupem času a se získáváním relevantních důkazů se z inovativních léčebných přístupů stávají standardní léčby. Níže je diskutován potenciál jednotlivých léčebných modalit, z nichž některé již můžeme v dospělé onkologii považovat za dnes standardní, nicméně data pro jejich použití v dětské onkologii chybí, jsou limitovaná nebo nekonkluzivní. Ve světle výše uvedeného se čeká na nové metodologické přístupy u „ultra orphan diseases“ a pomalu se ukazující důkazy i v malých souborech fází I a II klinických hodnocení.

1.5 „Drug repurposing“

Princip známý také jako „drug repositioning“. Jde o nalezení nové indikace k podání léčiva, které bylo původně schváleno k použití u jiného onemocnění. V klinické onkologii je jedním z nejznámějších valproová kyselina, valproát užívaný 50 let jako antiepileptikum. Byla zjištěna jeho schopnost inhibice histondeacetyláz, což ovlivňuje expresi genů ovlivňujících buněčný cyklus, diferenciaci a apoptózu i protinádorovou imunitu. Na jedné straně je z *in- vitro* studií

znám, že valproát na buněčné úrovni indukuje diferenciaci T-regulačních lymfocytů, které mají imunosupresivní účinek, využitelný např. při autoimunitních onemocněních, na druhou stranu existuje klinická evidence o jeho účinku na remodelaci chromatinu. Valproová kyselina přímo inhibuje histondeacetylázy (HDACs). Histony jsou v současnosti považovány z důležité aktéry epigenetické regulace prostřednictvím kovalentních modifickací na jejich N-terminálních koncích, které jsou na povrchu nukleosomu, což jim umožňuje interagovat s jadernými transkripčními faktory. Tento fenomén se jmenuje „histonový kód“ (angl.“histone code“) a jde o modifikace jednoho nebo více histonů tak, aby byl umožněn nebo naopak odmítnut přístup k transkripčním faktorům a regulačním proteinům, které modifikují proces aktivace nebo deaktivace genů, aniž by byl změněn genotyp. Valproát indukuje epigenetickou inhibici HDACs, čímž přispívá k vyšší acetylaci histonů H2, H3 a H4, které modifikují expresi genů asociovaných s apoptózou, buněčným cyklem, buněčnou diferenciací a protinádorovou obranou. Metabolomický efekt valproátu byl prokázán u AML, kdy jeho podání s nízce dávkovanou chemoterapií antimetabolity vedl ke změně metabolitů aminokyselin a mastných kyselin v séru. Recentně jsou publikovány práce, které prokazují, že podání valproátu je také spojeno se stimulací mechanismů buněčné imunity podmíněné protilátkami, tzv. ADCP a ADCC mechanismy. Toho lze využít při konkomitantím podávání s cílenými protilátkami jako je anti HER-2 trastuzumab, anti VEGF bevacizumab a další.

1.6 Léčebné modality

1.6.1 Tyrozinkinázové inhibitory

Tyrozinkinázové inhibitory (TKI) blokují signální dráhu, která je aktivována pomocí příslušné fosforylované tyrozinkinázy. U některých maligních onemocnění je přítomen fúzní gen podmiňující patogenezi nemoci a jeho produkt ve formě konstitutivně aktivované tyrozininázy je na inhibitor citlivý.

Skupina léčiv tyrozinkinázových inhibitorů je široce používána v dospělé onkologii. Jejich uvedení do praxe znamenalo značný přínos pro kvalitu života pacientů. Dříve používaná paliativní chemoterapeutická intravenózní léčba, která znamenala četné komplikace a nutnost

hospitalizací, doprovázená značnou morbiditou, byla vystřídána velmi dobře tolerovanou perorální léčbou. V dospělé hematoonkologii znamenala průlom, kdy se z dříve nezvladatelné choroby – např. CML – stala chronická nemoc pod kontrolou TKI s relativně dobrou kvalitou života.

V dětské onkologii je rozšíření TKI menší. Pokud se s nimi setkáme, tak nejvíce u hematologických malignit s definovanými fúzními geny. Limitem TKI je v průběhu léčby vznikající rezistence a je nutné jednotlivé TKI měnit za preparáty dalších generací. U solidních nádorů dětského věku byl v roce 2001 prvním FDA schváleným preparátem imatinib mesylát jako účinný u gastrointestinálního stromálního nádoru (GIST), následovaly sunitinib a regorafenib v roce 2013. U refrakterních sarkomů existuje terapeutická indikace pro pazopanib, který prodloužil dobu do progrese o 3–4 měsíce, objektivní radiologickou odpověď je možné čekat pouze ve 4 % případů. Pokud pacient na léčbu odpoví, je medián odpovědi 9 měsíců.

Velkou pozornost zasluhuje dávkování TKI. Zatímco standardně je k dávkování přistupováno se stejnou filozofií jako u chemoterapie, tj. v klinických hodnoceních byla testována maximálně tolerovaná dávka léčiva, v posledních letech přibývají důkazy, že snížená dávka léčiva až na 50 % i méně je stejně efektivní pro udržení v indukci navozené remise. V roce 2018 byl tento přístup matematicky modelován na základě dat ze dvou klinických studií fáze III u CML. V roce 2020 byl tento přístup validován u skupiny pacientů s CML, kteří byli léčeni redukovanými dávkami TKI (o padesát a více procent) a měli dobrou odpověď, a u nichž takové snížení dávky nevedlo ke zhoršení doby remise bez léčby ve srovnání s pacienty užívajícími standardní dávky TKI. Toto zjištění má velký potenciál v kombinované léčbě TKI a je nutné je validovat i u jiných onemocnění. V dětské onkologii otevírá tento přístup potenciál, který zatím není zkoumán, resp. přetrvává dogma podávání MTD. Na základě výsledků vysoké konkomitantní toxicity chemoterapie a standardních dávek TKI není toto dogma udržitelné a bude nutné ověřovat tyto kombinace i v nižších dávkách.

1.6.2 Check-point inhibitory

Nádorová imunosuprese hraje jednu z klíčových rolí v multifaktoriální patogenezi malignity. Určující pro rezistenci nádoru k léčbě je nejen samotná nádorová buňka, ale komplex mechanizmů, kterými nádor uniká z kontroly imunitního systému hostitele. Maligní nádor

a nádorové mikroprostředí, jako heterogenní směs různých buněčných populací a nádorem produkovaných imunsupresivních cytokinů, jsou cílem inhibitorů drah CTLA-4 a PD-1. Check-point inhibitory jsou používány od března 2011, kdy byl k použití schválen preparát ipilimumab pro léčbu metastatického melanomu. O rozvoji této formy imunoterapie svědčí množství prací, které jsou dohledatelné v PubMed Central – https://www.ncbi.nlm.nih.gov/pmc/ – kdy heslo „ipilimumab“ je nalezeno v 17658 případech (19. prosince 2020). Při pátrání po kombinaci „ipilimumab“ a „child“ bylo nalezeno 669 článků.

Teoretickým východiskem léčby check-point inhibitory je exprese CTLA-4 nebo PD-1 a PD-L1 antigenů v maligním nádoru. PD-L1 exprese byla nalezena u různých typů dětských nádorů – u Hodgkinových lymfomů, difusního velkobuněčného B-buněčného lymfomu nebo gliomů. U maligního melanomu existuje korelace mezi účinností monoterapií blokádou PD-1 nebo PD-L1 a vysokou mutační náloží (TMB-H). Kombinovaná terapie anti CTLA-4/anti PD-1/anti PD-L1 protilátkami je účinná, aniž by nutně korelovala s výší TMB-H jako u monoterapie.

Pembrolizumab

Pembrolizumab je monoklonální protilátka proti PD-1. U dospělých je schválena k použití ve 20 indikacích a stále přibývají další. U dětí byla zkoumána v monoterapii u pokročilých sarkomů měkkých tkání a skeletu, prokázala objektivní odpověď u 18 % sarkomů měkkých tkání a 5 % sarkomů skeletu. V současnosti je u dětí schváleno použití pouze u refrakterního Hodgkinova lymfomu po třech nebo více liniích předchozí léčby.

Ipilimumab

Humanizovaná monoklonální IgG1 protilátka proti CTLA-4. Klinická účinnost byla potvrzena u pokročilého maligního melanomu s jednoletým celkovým přežitím 45,6 %. U dětí nad dvanáct let je její použití schváleno od roku 2017 ve stejné indikaci jako u dospělých.

1.6.3 Jiné protilátkové imunoterapie

Blinatumomab – bispecifická protilátka proti znaku CD19, který je exprimován prakticky na všech B-buněčných akutních lymfoblastických leukemiích a lymfomech. FDA schválení pro léčbu relabovaných nebo refrakterních ALL získal v r. 2016.

Dinutuximab je chimérická humanizovaná protilátka proti glykolipidu GD2, který je exprimován na většině neuroblastomů a na některých dalších embryonálních nádorech, jako rabdomyosarkoma a Ewingův sarkom, pak i u osteosarkomů nebo některých gliomů. U neuroblastomu prokázala kombinace dinutuximabu s GM-CSF a IL-2 přidaná ke standardní léčbě isotretinoinem lepší dvouleté přežití bez události i celkové přežití. Tato registrační studie COG vedla ke schválení použití dinutuximabu v roce 2016.

1.6.4 Buněčné terapie

Tisagenlecleucel

Jde o chimérický antigen receptor (CAR)-T buněčnou terapii, která využívá autologní pacientské T-lymfocyty, které jsou geneticky modifikovány ve vazebné extracelulární antigen rozpoznávající doméně k cílené vazbě na CD19 antigen. Zpětné podání pacientovi vede k *in-vivo* tisícinásobné expanzi této T-lymfocytární populace s následnou perzistencí po dobu několika měsíců. V pilotní studii fáze I/II byla podána dvěma pacientům, v následných studiích s několika desítkami pacientů vedla léčba k celkové odpovědi a půlročnímu přežití bez události u přibližně 70 % pacientů. Všichni pacienti, kteří na léčbu odpověděli, měli toxicity související s uvolněním cytokinů a B-buněčnou aplazií, při které je nutná IgG substituce. Regulační autority schválily tuto léčbu v roce 2017.

Protinádorové vakcíny

Rozvoj protinádorových vakcín je patrný na více úrovních. Technologie přípravy je široká, od buněčných vakcín, které obsahují nádorové lyzáty, přes vakcíny používající specifické nádorové peptidy jako cíl indukované simulace dendritických buněk, po ty, které pracují s induktorem imunitní odpovědi DNA nebo RNA nádoru nebo vakcíny využívající virového vektoru.

Překvapivě dobré jsou výsledky kombinace nízko dávkovaného cyklofosfamidu a alogenní nádorové vakcíny v kombinaci s GM-CSF u relabovaných neuroblastomů. Jiný přístup zvolili výzkumníci na University of Florida u vysoce maligních gliomů, kdy kombinují dendritické buňky s alogenní gliomovou RNA spolu s aplikací GM-CSF, ve druhém kroku je pacientovi podána infuse tumor-specifických T-lymfocytů. Tato studie byla zahájena v roce 2017 a zatím nejsou známy výsledky.

U medulloblastomů a vysoce maligních gliomů je používána peptidová vakcína derivovaná z CMV, který je znám jako spouštěč onkogeneze u některých gliomů. U dospělých pacientů vedla k významnému prodloužení přežití bez události i celkového přežití.

1.6.5 Neschválené terapie ve fázi klinického vývoje

Onkolytické viry

Jde o buď nepatogenní „wild type“ viry nebo atenuované geneticky modifikované viry, které působí přímo cytotoxicky protinádorově nebo nepřímo stimulují protinádorovou imunitu. Ve fázi I klinického zkoušení byla prokázána bezpečnost použití modifikovaných virů herpes simplex a vakcinie u extrakraniálních nádorů dětského věku, i když při podané dávce nebyla pozorována objektivní odpověď. Jedinou FDA schválenou léčbou na bázi virové terapie je talimogene laherparepvec (T-VEC), atenuovaný herpesvirus typu 1 exprimující gen pro lidský GM-CSF v indikaci pokročilého melanomu. V kombinaci s pembrolizumabem prokázal vysoký potenciál pro dosažení léčebné odpovědi u pokročilého melanomu

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NK buňky

Na rozdíl od T-lymfocytů nepotřebují NK buňky (lymfocyty) předchozí stimulaci nádorem, aby byly aktivní. Jejich účinek je přímo cytotoxický a indukuje u nádorové buňky apoptózu. Aktivace NK buněk v nádorovém mikroprostředí se zdá být podmínkou pro migraci dendritických buněk a T-lymfocytů do nádoru. Mohou být separovány z periferní krve, pupečníkové krve, a v případě nutnosti početně expandovány *ex-vivo*. Jejich použití je bezpečné a dobře tolerované. Protinádorová účinnost je ovšem limitována pravděpodobně supresivním efektem nádorového mikroprostředí.

Hlavními imunosupresivními faktory v nádorovém mikroprostředí jsou transforming growth factor beta (TGFβ), indoleamine 2,3-dioxygenase (IDO) a IL-10. Existují postupy genového inženýrství, které pomocí technologie CRISPR dokážou indukovat větší odolnost NK buněk vůči imunosupresivnímu působení cytokinů. CAR-NK manipulované anti CD19 buňky vykazují vysokou protinádorovou aktivitu proti B buněčným leukemiím.

Velký potenciál NK buněčné terapie je v kombinaci jednotlivých typů imunoterapie.

1.6.6 Antiangiogenní strategie

Klíčovým mediátorem angiogeneze je VEGF. Existují četné anti VEGF protilátky nebo tyrozinkinázové inhibitory, které jsou používány v klinické praxi. Jejich kombinace s imunoterapií je atraktivním přístupem, protože blokáda VEGF ipilimumabem vede k synergistickému efektu u pacientů s metastatickým melanomem. V preklinickém modelu xenograftu neuroblastomu byl prokázán synergistický efekt při kombinaci anti VEGF protilátky bevacizumabu a anti GD-2 CAR-T buněk. I nízké dávky bevacizumabu v této kombinaci vedly k efektu, který byl v monoterapii zanedbatelný.

Antiangiogenní léčba vede k větší infiltraci a aktivaci imunokompetentních dendritických buněk do nádoru, spolu s redukcí imunosupresivních MDSC.

1.7 Inovativní léčebné postupy na Klinice dětské onkologie LF MU a FN Brno

1.7.1 Imunoterapie dendritickou vakcínou

V roce 2013 byla na KDO FN Brno zahájena práce na realizaci klinického hodnocení KDO DC 1311s názvem „Kombinovaná protinádorová terapie s *ex vivo* manipulovanými dendritickými buňkami produkujícími interleukin-12 u dětských, adolescentních a mladých dospělých pacientů s progredujícími, relabujícími nebo primárně metastatickými malignitami vysokého rizika“, EudraCT No. 2014-003388-39. Vycházela z předchozí zkušenosti výzkumného týmu ACIU LF MU s výrobou nádorového lyzátu pro přípravu autologní vakcíny z dendritických buněk u dospělých pacientů s renálním karcinomem. Tento projekt, původně plánovaný v jiném složení řešitelů, nebyl dokončený pro legislativní a technologické změny – nutné pro legální a správnou klinickou praxi respektující postupy. Zpoždění v náboru pacientů vedlo k zastavení grantové podpory. Ovšem významné technologické pokroky a nastavení kvality výroby díky tomuto nedokončenému projektu umožnilo plánování a realizaci studie u dětských onkologických pacientů. Nábor pacientů byl v projektu KDO DC 1311 dostatečný. Vyhodnocení jednotlivých cílů doposud probíhá. Publikované práce reflektují výsledky primárních cílů studie. Vedle prokázání bezpečnosti výroby vakcíny je podstatné zjištění o

vyvolání měřitelné imunitní odpovědi, což může sloužit jako biomarker pro budoucí vývoj vakcíny.

Analýza klinické účinnost je plánována na rok 2021, aby tak bylo možné vyhodnotit sekundární cíle výzkumu s adekvátním časovým odstupem.

Proces výroby vakcíny začíná identifikací pacienta vysokého rizika, jehož šance na přežití jsou méně než 30 % ve 3 letech od diagnózy nebo relapsu. Po podepsání informovaného souhlasu je prvním krokem k výrobě vakcíny odebrání nádorové tkáně. Ve většině případů jde o diagnostickou nebo terapeutickou indikaci operačního výkonu, a nejedná se tedy o indikaci z důvodu inovativní terapie, ale naopak odběr na výrobu vakcíny je spojen s těmito indikacemi. Odběr tkáně na výrobu léčiva somatobuněčné terapie podléhá zákonným požadavkům na zacházení s lidskými tkáněmi a buňkami, proto je nutné splnit přísná kritéria kvality a příslušné odběrové místo musí mít povolení státních autorit. Toto povolení má v současné době (r. 2020) Fakultní nemocnice Brno. Odebraná nádorová tkáň je zpracována v čistých prostorách ACIU na formu nádorového lyzátu. Tento lyzát je uschován k dalšímu použití a zároveň probíhá kontrola mikrobiologické čistoty. Druhou fází výroby je odběr autologních monocytů pacienta. Tento odběr probíhá po zavedení centrálního venosního katetru na separátoru. Cílem separace je odběr minimálně 0,5 x 10^9 monocytů. Takto odebrané monocyty jsou dále ve výrobě zpracovávány s nádorovým lyzátem, cílem je indukce reaktivity monocytů derivovaných dendritických buněk proti nádoru, mechanisticky proti nádorovým neoantigenům.

Dosavadní zjištění v publikované práci (příloha č. 1) se zabývá výrobním procesem dendritické vakcíny. Identifikovali jsme léčebné postupy před odběrem autologních monocytů, které vedou k horší výtěžnosti vakcíny a horší maturaci dendritických buněk a produkci IL-12. Nastavené parametry kontroly kvality pak nedovolí takovou vakcínu podat pacientovi. Chemoterapeutika cyklofosfamid a topotecan a tyrozinkinázový inhibitor pazopanib vedou k poruše diferenciace a poté inadekvátním imunostimulačním vlastnostem dendritických buněk. Kombinace temozolomidu a irinotecanu sice dovolí diferenciaci monocytů, ale výsledné dendritické buňky nemají adekvátní imunostimulační vlastnosti. Tato zjištění vedla ke změnám v logistice separace monocytů v návaznosti na druh podané léčby. Od listopadu 2017 byla zavedena pravidla odstupu separace monocytů od podané léčby. Tyrozinkinázové inhibitory musely být vysazeny s odstupem závisejícím na jejich biologickém poločasu: léčiva s krátkým poločasem, 3–14 hodin, nejméně 2 dny před leukaferézou (axitinib, dabrafenib, dasatinib, ibrutinib,

idelalisib, nintedanib, ruxolitinib, trametinib), léčiva se středním poločasem, 15–35 hodin, nejméně 7 dní před leukaferézou (alectinib, bosutinib, lapatinib, lenvatinib, nilotinib, osimertinib, pazopanib, ponatinib, regorafenib a mTOR inhibitor everolimus) a léčiva s dlouhým poločasem, 36–60 hodin, nejméně 12 dní před leukaferézou (afatinib, ceritinib, erlotinib, gefitinib, imatinib, cabozantinib, crizotinib, sorafenib, sunitinib, vemurafenib a mTOR inhibitor temsirolimus). Myelopoetické růstové faktory byly vysazeny nejméně 7 dní před leukaferézou. Následně se podařilo zrychlit proces mikrobiologické kontroly kvality lyzátu a v současnosti je možné po několika dnech od odběru nádorové tkáně provést i odběr monocytů. Těmito postupy se můžeme vyhnout negativním vlivům podávané protinádorové léčby na monocyty pacienta. Efektivita výroby vakcíny se tím významně zlepšila.

Druhá publikovaná práce (příloha č. 2) se zabývá vlivem vakcinace dendritickými buňkami na imunologické parametry pacientů se sarkomy a popisuje jeden případ ilustrující validitu naměřených parametrů na klinickém průběhu nemoci. Kvantitativní parametry imunity byly hodnoceny při každém podání protinádorové vakcíny. Navíc bylo provedeno funkční testování odpovědi pacientových T-lymfocytů na autologní nádorový lyzát v tzv. auto-MLR reakci. V práci je uveden případ pacienta s diagnózou metastatický relabující Ewingův sarkom. Pacient prodělal dva relapsy onemocnění a bylo mu podáno 19 dávek vakcíny po prvním relapsu, spolu s konkomitantní onkologickou léčbou bylo dosaženo parciální remise. Opětovná revakcinace po druhém relapsu vedla ke stimulaci preexistující odpovědi na nádorové antigeny a T-buněčná reaktivita (měřeno auto-MLR reakcí) perzistovala i po předchozí vakcíně a byla zvýšena po revakcinaci. Tato zjištění představují zásadní vhled do imunologických mechanismů vyvolaných dendritickou vakcínou a jsou základem pro další vývoj této léčebné metody.

1.7.2 Personalizovaná léčba

Využití personalizované léčby je publikováno ve dvou pracích. První z nich ukazuje využití molekulární diagnostiky u pacientů s Burkittovým lymfomem (příloha č. 3). Vyšetření pomocí celoexomového sekvenování nádorové tkáně, vyšetření transkriptomu a aktivity tyrozinkináz vede k identifikaci pacientů, jejichž nádor je cílitelný v současnosti dostupnými léčivy. V práci jsou popsány nálezy u tří pacientů, jejichž nádory s totožnou histologií mají zcela rozdílné biologické profily měřené uvedenými metodami. Ukazuje se, že klonální evoluce vedla u jednoho pacienta k TP53 mutaci a k chemorezistenci. Na základě molekulárního profilu

germinální mutace PI3K-delta, transkriptromikou zjištěné expresi HR23B, která je prediktorem účinnosti HDACs, byla pacientovi podána léčba PI3K inhibitorem idelalisibem spolu s ibrutinibem a valproátem, na základě exprese PD-1 v nádorové tkáni byl přidán nivolumab a personalizovaná dendritická vakcína. Tato kombinovaná léčba vedla u pacienta k navození a udržení kompletní remise, která byla v době publikace nejdelší z jeho tří intervalů přežití bez progrese. Jiný pacient dosáhl remise na základě indikace imunoterapie nivolumabem „off-label“ při vysoké mutační náloži TMB-H 31 mutací/Mb.

V druhé práci se autoři zabývají mutační náloží u 106 pacientů s 28 různými histologickými diagnózami (příloha č. 4). Jde o metodickou práci srovnávající vyšetření mutační nálože pomocí dvou různých metod – celoexomového sekvenování a standardizovaného panelu vyšetření mutační nálože, FoundationOne Heme. Byla nalezena významná variabilita výsledků, které závisí na použitých algoritmech analýz a laboratorních metodách. Práce přispívá svými výsledky k aktuální diskusi o zavedení harmonizované metodiky testování pro použití v klinických hodnoceních, což je důležité ke srovnatelné interpretaci výsledků léčby „checkpoint“ inhibitory. Jejich účinnost je v silné korelaci s mutační náloží nádoru.

Přehledová práce na téma personalizované medicíny byla publikována ve spolupráci s řadou zahraničních spolupracovníků (příloha č. 5). Zabývá se tématem změny paradigmatu v onkologii s posunem k precizní a personalizované medicíně. Předpokladem k efektivnímu využití stávajících léčiv v jiných než schválených indikacích („drug repurposing“) nebo nových léčiv použitých bez ohledu na tkáňovou diagnózu (agnostický přístup) je přijetí principu „N-of-1 trials“, který se zásadně liší od typického klinického zkoušení ve fázích I–III. Tento nový přístup vychází z rychle se rozvíjejících poznatků nádorové biologie a postupuje dříve nevídanou rychlostí, se kterou se léčivo může k pacientovi dostat. Klasické paradigma zkoušení nového léčiva u tkáňově definované skupiny pacientů nezohledňuje individuální nádorovou biologii a biomarkery a zřídka vede k úspěchu. Nové paradigma konceptu klinických hodnocení naopak postupuje cestou definování biomarkerů, cílů terapie a zkoumání účinnosti léčiva na takto definované skupině pacientů, mnohdy bez ohledu na histologii. Metodologicky tento přístup využívá nové matematické modelování a je natolik komplexní, že není v silách „běžného“ klinika pochopit všechny jeho detaily. Zcela nezbytným se stává multidisciplinární přístup k diagnostice a návrhu terapie, kdy čím dál větší vliv v rozhodování o léčebném postupu mají molekulární biolog a klinický farmakolog. Nové jsou také kombinované léčby, které ve větší či menší míře zakomponují do léčby první linie genomické a biologické informace a vedou k individualizaci léčby. Mnohdy je kontraproduktivní podávat monoterapii cíleným léčivem

bez znalosti tkáňové biologie nádoru (nepersonalizované cílená terapie, „non-personalized targeted therapy“) a recentně se objevují důkazy, že takový přístup může vést k horším výsledkům než klasická cytotoxická terapie. Také kombinace maximálně tolerovaných dávek chemoterapie s přidaným cíleným léčivem je zpravidla kontraproduktivní, s vyšší toxicitou a bez lepší léčebné odpovědi.

1.7.3 Antiangiogenní a metronomická léčba

Léčba byla složena z kombinované antiangiogenní strategie COMBAT („combined oral maintenance biodifferentiating and antiangiogenic therapy“). V této strategii je používáno více antiangiogenních a imunomodulačních prvků podávaných metronomicky v nízké dávce trvale po dobu několika let – nízko dávkovaný cyklofosfamid, temozolomid, topotecan nebo vepesid, vinka alkaloid vinblastin nebo vinorelbin, COX-2 inhibitor celecoxib a variabilně hypolipidemikum fenofibrát, antiangiogenní bevacizumab a další. Komentované publikace na toto téma popisují dobrou toleranci léčby. Největšími benefity jsou ambulantní podávání umožňující běžné denní aktivity a absence toxicit, které by vyžadovaly hospitalizace. Pacienti na této léčbě běžně chodí do školy. Benefitem je také udržení celkového stavu pacienta, měřeno Lanského nebo Karnofského škálou.

První generace protokolů COMBAT byla složena z nízko dávkovaného vepesidu a temozolomidu, celexocibu a biodiferenciační cis-retinové kyseliny podávané po dobu jednoho roku v jedenáctitýdenních cyklech léčby. Na souboru 22 pacientů z jednoho centra autoři publikovali benefit pro pacienta ve formě stabilizace onemocnění (příloha č. 6). Toxicita této kombinace se týkala především cis-retinové kyseliny ve formě cheilitid u 7 pacientů, hematologická toxicita stupně 3 se vyskytla u jednoho pacienta. Při zaznamenání takové toxicity byla v dalším cyklu redukována dávka chemoterapeutika. Ze 14 dětí s progredujícím onemocněním, u kterých bylo možné hodnotit léčebnou odpověď, byla zaznamenána u 6 dětí, u 3 pak stabilizace nemoci, odpovídající celkovému benefitu pro pacienty 64 %.

V další práci byla publikována zkušenost s protokolem COMBAT jak na KDO FN Brno, tak v zahraničí na spolupracujících pracovištích v Košicích na Slovensku a v Marseille ve Francii (příloha č. 7). Změnou oproti původní verzi protokolu bylo přidání fenofibrátu a vitamínu D do metronomického schématu a prodloužení doby léčby na dva roky, vznikl protokol COMBAT II. Verze pro měkkotkáňové sarkomy se nazývala COMBAT IIS, ve kterém byl etoposid

s temodalem nahrazen nízce dávkovaným cyklofosfamidem, a byl přidán intravenózně vinorelbin. Pacienti vysokého rizika a bez možnosti kurativní léčby byly do této studie zařazeni od roku 2004 do roku 2010. Celkově se léčby zúčastnilo 74 pacientů. Klinický benefit pro pacienta byl zaznamenán ve 40 % případů. Toxicita léčby byla podobná, jak bylo publikováno v předchozí práci, zaznamenány byly především cheilitidy a zvýšené hodnoty jaterních testů.

V další verzi protokolu COMBAT III byla cis-retinová kyselina nahrazena bevacizumabem, anti VEGF protilátkou podávanou ve dvoutýdenních intervalech.

S nástupem tyrozinkinázových inhibitorů a rozvojem měření aktivity tyrozinkináz nebo MAP kináz byla do schématu COMBAT zakomponována i tato léčba-COMBAT III modif. Důležitá je titrace dávek léčiv tak, aby nedocházelo k neutropeniím vyžadujícím přerušení léčby nebo k dalším komplikacím, které vyžadují hospitalizaci. Data o účinnosti jsou aktuálně zpracovávána, do doby psaní tohoto textu je zpracována kohorta pacientů se sarkomy. Rámcově lze říct, že léčba COMBAT III modif. vede k prodloužení doby přežití u vysoce rizikových sarkomů. Konkomitance nízce dávkované chemoterapie se zakomponováním intravenózního vinblastinu s antiangiogenním účinkem a tyrozinkinázových inhibitorů je dobře snášena. Vyhodnocení efektivity u dalších diagnostických skupin je v plánu.

1.7.4 Tyrozinkinázové inhibitory

Velká část klinického výzkumu efektivity tyrozinkinázových inhibitorů u solidních nádorů probíhá bez znalosti aktivace příslušných kináz v nádoru jednotlivého pacienta, tedy tzv. nepersonalizovaný cílený přístup. Vhodnější se zdá být přístup, kdy je jako biomarker měřena buď exprese tyrozinkinázy pomocí imunohistochemického histopatologického vyšetření, nebo některá z molekulárních metod pro určení aktivity příslušné tyrozinkinázy, např. aktivační mutace v kódujících exomech nebo na úrovni funkce proteinu podle míry jeho fosforylace (aktivovaný stav).

Samostatné podání monoterapie tyrozinkinázovým inhibitorem je předmětem publikované práce (příloha č. 8). Pojednává o novorozenci, u kterého byly nalezeny mnohočetné měkkotkáňové, orgánové a kostní léze diagnostikované jako infantilní myofibromatóza. Konvenční léčba byla zatížena vysokou toxicitou s nutností modifikace dávek chemoterapie. Léčebná odpověď – parciální remise po této léčbě vydržela pouze tři měsíce. Vyšetřením

nádorové biologie byla zjištěna vysoká konstitutivní aktivita (fosforylace) tyrozinkinázy PDGFRβ. Dalším vyšetřením jsme zjistili nález aktivační mutace v kódujícím genu PDGFRB. Tato zjištění vedla k podání personalizované cílené léčby („personalized targeted therapy“) s excelentním výsledkem, kdy v řádu týdnů došlo k regresi myofibromatózy a i několik let od zahájení léčby pacient pokračuje v terapii; byť s nutností redukce dávek tyrozinkinázového inhibitoru a s několika infekčními komplikacemi a jednou symptomatickou hypoglykemií s nutností hospitalizace. Evoluční vývoj onemocnění vedl při redukci dávek k několika relapsům, které ovšem během času vyhasínaly, při zachování alespoň malé dávky tyrozinkinázového inhibitoru. Zajímavostí bylo, že pacientova 8letá sestra měla v anamnéze spontánně regredované nebioptované léze a v době léčby chlapce u ní došlo k novému vzplanutí choroby s relativně velkým ložiskem na bazi lební s velkými bolestmi. Biologie nádoru byla stejná jako u chlapce a stejně tak bylo velmi rychle dosaženo terapeutického úspěchu s tyrozinkinázovým inhibitorem sunitinibem. Tato práce ilustruje, jak velkým benefitem je pro pacienta personalizovaná cílená léčba. V případě nálezu aktivační mutace je možné podání inhibitoru v monoterapii s velmi dobrým výsledkem léčby, jak je v práci dokumentováno.

Z tkáně nádoru pacienta popsaného v předchozím případě byla vytvořena buněčná linie, u které byla zkoumána fosforylace tyrozinkináz a citlivost na různé tyrozinkinázové inhibitory (příloha č. 9). V práci bylo experimentálně potvrzeno, že i buněčné linie derivované z nádoru si udržují vysokou míru fosforylace různých tyrozinkináz, nejvíce pak PDGFRβ podmíněnou mutací v genu PDGFRB. Inhibiční efekt sunitinibu byl zaznamenán při koncentracích, které jsou v klinické praxi dosažitelné. Fosforylace PDGFR nebyla podmíněna jenom přítomností aktivační mutace, ale v nepřítomnosti séra v kultivačním médiu došlo ke snížení exprese genu pro kinázu TGFA, nezměnila se exprese EGFR a PDGFRB, a překvapivě došlo ke zvýšení exprese genu PDGFRA. Jde pravděpodobně o adaptační autokrinní mechanismus pro přežití buňky při nedostatku živin. Potenciálně je tento mechanismus využitelný při kombinované terapii v klinické praxi.

Případem úspěšného požití tyrozinkinázového inhibioru v monoterapii je případ batolete s vzácným onemocněním „fibrodysplasia progressiva ossificans“, FOP (příloha č. 10). Dvacetiměsíční dívenka přišla k lékaři s ložiskovými zarudnutími, otoky krku, axily a jugula, některé z nich po prvotním zarudnutí spontánně mizely, jiné se objevovaly, léze vedly k omezení mobility krku a pletence horní končetiny. Histologické vyšetření popsalo infantilní (lipo)fibromatózu měkkých tkání, při stagingu byly objeveny další asymptomatické léze na hrudníku a zádech. Byla zahájena léčba standardní nízkodávkovanou chemoterapií

methotrexate/vinblastin. Po čtyřech týdnech ovšem došlo k progresi lézí a objevování se nových, ve velmi rychlém sledu (v řádu hodin) doprovázených teplotou. Kortikoidy vedly k omezení těchto vzplanutí. Vyšetření biologické aktivity nádoru odhalilo vysokou míru fosforylace PDGFRβ. Dívce byla podána léčba sunitinibem, který cílí na tuto kinázu, v kombinaci s nízkodávkovaným vinblastinem a celecoxibem. Na této léčbě došlo ke zmenšení lézí, omezení frekvence a intenzity vzplanutí a ke zpomalení progrese omezení hybnosti. Při vyšetření celoexomovým sekvenováním byla odhalena patogenní mutce v AVCR1 genu, která je patognomonická pro onemocnění FOP, čímž změnila histopatologickou diagnózu. Dívka je na této léčbě již čtyři roky, během kterých je schopna s omezením mobility krku a horních končetin běžných denních činnost. V tomto případě nejde o cílenou léčbu, mechanismem účinku je inhibice prozánětlivých cytokinů a proliferativních kaskád včetně PDGFR α, PDGFRβ, c-kit, HIF1α a dalších. Tato kazuistika ilustruje, jak metody molekulární genetiky přispívají ke správné diagnostice ultravzácných onemocnění, kterým FOP je, a jak „off-label“ použití léčiva vede k léčebnému efektu. V době zahájení léčby byla dívka nejmladší pacientkou v dohledatelné anglickojazyčné literatuře, která tyrozinkinázový inhibitor v této indikaci dostala.

1.7.5 Rekurentní laryngeální papilomatosa

Rekurentní papilomatosa hrtanu je onemocnění podmíněné lidským papilomavirem (HPV). Vyskytuje se ve všech věkových kategoriích. Cesta transmise viru je genitální. Vzácně se objevuje u malých dětí. V případě, že jde o onemocnění refrakterní na chirurgickou léčbu a opakovaně recidivuje, jde o devastující onemocnění, které pacientům nedovolí zapojení do běžného života. Pacienti trpí opakovanými ztrátami hlasu, nekonstantní barvou a zněním hlasu a vedou k psychosociální izolaci v kolektivu.

Do léčby rekurentní laryngeální papilomatosy bylo zavedeno více lokálních nebo systémových přístupů, ale žádný z nich nedosáhl velké efektivity. Inovativní přístup léčby tohoto onemocnění je publikován v práci, kde je ukázána efektivita použití očkování pro HPV (příloha č. 11). Dvouletý chlapec, jehož matka neměla žádné známky HPV, postupně začal mít chraplavý hlas a při vyšetření ORL specialistou byla diagnostikována laryngeální papilomatosa. Během následujících dvou let bylo provedeno šest lokálně chirurgických zákroků. Tato lokální léčba vedla k dočasným úlevám. Pomocí PCR byla v papilomu detekována genotypická

přítomnost HPV-11. U chlapce nebyla detekována žádná porucha imunity. Bylo proto uvažováno o navození imunity proti HPV-11 po očkování, která by zajistila dlouhodobější efekt než lokální nebo toxické systémové léčby. Vzhledem k etiopatogenezi byla použita specifická vakcína cílená proti více genotypům HPV, mezi nimi HPV-11. Protilátková odpověď byla dostatečná a u chlapce došlo k vytvoření protilátkové imunity. Po očkování nedošlo k další recidivě papilomatosy po dobu 17 měsíců (v době psaní článku), což byl do té doby nejdelší zaznamenaný interval bez nemoci, hlas se chlapci udržel ve fyziologických mezích tónů. V době publikace šlo o „off label“ použití a fakticky o „drug repurposing“ použití léčiva schváleného pro jiné indikace. Tato práce má velký citační ohlas a vakcinace proti HPV je v současnosti akceptována jako léčebná metoda a strategie pro toto onemocnění.

1.7.6 Agnostická protinádorová léčba

Jde o postup, kdy je léčba určena genetickým nebo molekulárním nálezem bez ohledu na typ nádoru. Dosavadní paradigma klinické onkologie hovořilo o klasifikaci histologické. S nálezem molekulárně genetických změn v rámci jednotlivých histologických typů nádoru dochází k dramatické změně nádorových klasifikací, typicky u medulloblastomu nebo ependymomů na základě metylačního profilování. U nádorů se stejným typem genové změny – mutace („gain of function“) nebo genové fúze lze nově indikovat cílenou léčbu.

U nemaligních onemocnění jsme se spoluautory zjišťovali přítomnost mutací v genech TEK2 a PI3CA u vaskulárních malformací. Ty mohou pacienta prakticky invalidizovat a významně mu zhoršit kvalitu života. Zjištěné mutace jsme pak korelovali s klinickou odpovědí na alpelisib. Léčivo je schválené v indikaci PIK3CA mutovaný karcinom prsu. Při použití „off-label“ jsme zaznamenali významné zlepšení jak laboratorních parametrů onemocnění (pokles D-dimerů), objektivní odpověď na zobrazovacích metodách i klinickou úlevu od bolestí nebo otoků. (příloha č. 20).

1.8 Souhrn

Inovativní léčebné postupy imunoterapie, personalizované léčby, antiangiogení metronomické terapie, tyrozinkinázových inhibitorů a vakcinací byly dokumentovány přiloženými pracemi.

V klinické praxi vedly k rozšíření možností léčby maligních i jiných onemocnění, jako rekurentní laryngeální papilomotosa nebo „fibrodysplasia ossificans progresiva“. U malignit šlo v některých případech o kurativní přístup u do té doby beznadějných případů, jiným pacientům dokázala inovativní léčba alespoň udržet celkově uspokojivý stav po delší období, a to bez nutnosti hospitalizací nebo nežádoucích účinků, a významně tak přispěla ke kvalitě života pacientů.

2 Antimykotická léčba u imunokompromitovaných pacientů

2.1 Úvod

Mykotické infekce jsou závažnou komplikací u pacientů léčených pro maligní onemocnění, především hematoonkologické. V dospělé onkologii se lze s mykotickou infekcí setkat vzácně. U hematoonkologických pacientů nebo dětských onkologických pacientů s vyšší intenzitu chemoterapie je četnost mykotických onemocnění vyšší. Nejrizikovější jsou pacienti s akutní myeloblastovou leukemií a pacienti po alogenní transplantaci kostní dřeně s kombinovanou imunosupresí a s nemocí štěpu proti hostiteli (GvHD).

Historicky je prvním velmi účinným antimykotikem amphotericin B. Byl široce používaným přípravkem desítky let jako zlatý standard antimykotické léčby. Jde o makrocyklické polyenové protiplísňové antibiotikum produkované bakterií *Streptomyces nodosum*. V době jeho objevení v r. 1959 šlo o život zachraňující lék, k jeho používání nebyla nutná žádná randomizovaná studie. Naopak, dlouhá léta byl používán jako komparátor nově registrovaných a objevovaných antimykotik, jak echinokandinů, tak azolů, v pozdějším období pak byly stejně využívány jeho lipidové formy. Velkou nevýhodou konvenčního amphotericinu B je nefrotoxicita, která byla limitující pro dlouhodobé podávání. Proto byly na trh uvedeny lipidové formy, které mají menší afinitu k cholesterolu membrán na lidských buňkách a zároveň mají zachovánu afinitu k ergosterolu membrán hub. Na trh byly uvedeny tři formy – liposomální forma (Ambisom), lipidový komplex (Abelcet) a koloidní disperze (Amphocil). Všechny tři mají dobrou renální toleranci, která umožňuje dlouhodobé podávání. Spektrum účinnosti amphotericinu B sahá od candid, kryptokoků, blastomycet přes *aspergillus spp*. až po mukormycety. Z vlastní praxe autora je možné podávání těchto lipidových forem výjimečně i několik měsíců bez signifikantní toxicity, jako u pacientů s invazivní aspergilózou mozku.

V posledních dvou desetiletích byla ke klinickému použití vyvinuta echinkandinová a nová azolová antimykotika. Jejich zavedení do praxe znamenalo rozšíření spektra účinných antimykotik. U aspergilózy byl nastaven nový standard terapie voriconazolem, v případě candidových infekcí je lékem volby echinokandin. Velkým přínosem je menší nebo žádná renální toxicita, malá hepatální toxicita a u azolů možnost perorální formy. V praxi se uplatňují jak v léčbě mykóz, tak v profylaxi. Profylaktické podávání azolů je standardem u vysoce rizikových hematoonkologických diagnóz jak u dospělých, tak u dětí.

V České a Slovenské republice je etablována síť spolupracujících hematoonkologických center. Jejich spolupráce je v oblasti antiinfekční léčby, koordinace léčebných postupů a společných databází mykotických onemocnění prováděna v rámci skupiny CELL – Czech Leukemia Study Group for Life, formálně jde o občanské sdružení. Klinika dětské onkologie FN Brno je jedním ze spolupracujících center. Cíli sdružení jsou tvorba společných diagnostických a léčebných protokolů, organizování klinických a experimentálních studií, zavádění nových poznatků do diagnostiky a léčby, navázání kooperace se zahraničními subjekty a rozšiřování poznatků i mezi laickou veřejnost (osvětová činnost). Zaměření tedy pokrývá celé spektrum hematoonkologické problematiky, nejen diagnostiku a léčbu mykóz u imunokompromitovaných pacientů.

Autoři z KDO FN Brno se podíleli na definování doporučených postupů léčby invazivních mykóz, které byly publikovány v českých časopisech. Představují souhrn do té doby roztříštěných časopiseckých prací, klinických studií a výsledků kooperativních mezinárodních skupin a navazují na podobná úsilí, která byla vyvinuta v zahraničí.

2.2 Komentář k publikovaným pracím

Množství dat o účinnosti a efektivitě voriconazolu u dětí je limitované. Pro rozšíření indikace voriconazolu na dětskou populaci byla provedeno klinické hodnocení (KH) voriconazolu u invazivní aspergilózy, invazivní kandidózy a esofageální kandidózy u dětí od 2 do 18 let (příloha č. 12). Na KDO probíhala dvě diagnóza specifická KH. První zkoumající voriconazol u invazivní aspergilózy (IA), NCT00836875, druhé v indikaci invazivní kandidózy a esofageální candidózy (IC/EC), NCT01092832. Obě KH byla multicentrická, sponzorována farmaceutickou firmou Pfizer, probíhala v 16 centrech v Evropě, Asii a Severní Americe v letech 2009–2013. Dávkování voriconazolu bylo nastaveno podle v té době nových znalostí o farmakokinetice, kdy u pacientů do 12 let nebo 12–14 let s hmotností pod 50 kg byla startovací dávka 9 mg/kg á 12 hodin první den, poté 8 mg/kg á 12 hodin, v případě esofageální kandidózy byla dávka 4 mg/kg. Bylo možno dávku modifikovat podle dosažených plazmatických hladin voriconazolu. Do studie IA bylo zařazeno 31 pacientů, do studie IC/EC bylo zařazeno 24 pacientů. Medián doby podávání antimykotika byl ve skupině IA 41 dní, u skupiny IC/EC 14 dní. Tolerance léčby byla dobrá. Celková léčebná odpověď byla zaznamenána u 78 % pacientů s IA ve věku 12–18 let, u mladších pacientů byla účinnost horší – 40 %, ale může jít o zkreslení vlivem malého vzorku, hodnocených pacientů bylo pouze pět.

Celková účinnost u IA byla 64,3 %. Profil toxicity byl podobný jako u dospělých, nicméně u mladších dětí byla zaznamenána častější hepatotoxicita, ale nikoliv zvýšení jejího stupně. Účinnost ve skupině pacientů s IC/EC byla lepší ve věkové skupině 2–12 let, 88,9 %, u starších byla 62,5 %, celkově 76,5 %. Interpretce těchto rozdílů mezi věkovými skupinami je limitována malou velikostí studované populace a faktem, že se jednalo o „open-label“ nekomparativní design studie. Celkově lze shrnout, že profil účinnosti a toxicity voriconazolu se v uvedených indikacích pro dětskou populaci neliší od dat získaných dříve u dospělých pacientů, a dovoluje indikaci tohoto azolu v dané věkové skupině.

O retrospektivním výzkumu léčby invazivní aspergilózy u hematoonkologických pacientů v České a Slovenské Republice v letech 2005–2009 pojednává práce publikovaná v r. 2012 (příloha č. 13). Data byla získána v rámci projektu CELL. Autoři popisují diagnostiku a léčbu u 176 případů IA. U 15,3 % byla diagnostikována prokázaná IA, u 84,7% pravděpodobná IA, definice vycházely z EORST/MSG kritérií pro IA z roku 2002. Kompletní nebo parciální léčebné odpovědi bylo dosaženo u 53,2 % pacientů. Důležitým zjištěním je fakt, že pouze 53,7 % pacient mělo na diagnostických CT vyšetřeních léze považované za typické pro plicní IA dle EORTC/MSG 2008 kritérií. Nález na CT je významně ovlivněn přítomností nebo absencí neutrofilních segmentů. Vliv v tomto zjištění mohl mít i fakt, že v době studie se do praxe dostávaly HRCT přístroje, a diagnostické protokoly se tak lišily od předchozích CT technik. Významným zjištěním v této práci je role testování galaktomananu jak v séru, tak v tekutině z bronchoalveolární laváže, který byl pozitivní v 79,1 % resp. 78,8 % případů, tyto pozitivity přispěly k diagnostice IA. U čtvrtiny pacientů (26,3 %) byla IA léčena dvojkombinací antimykotik, nejčastěji voriconazolu a capfunginu, ovšem bez korelace s léčebným efektem. V současné době se za standardní postup považuje monoterapie voriconazolem nebo posaconazolem nebo isavconazolem, které mají schválení EMA v této indikaci.

Velkým úspěchem byla léčba dvou invazivních mykóz publikovaných jako kazuistiky.

V prvním případě byl pacientce s aspergilózou mozku během indukční léčby akutní lymfoblastické leukémie podáván lipidový komplex ampfotericinu B („amphotericin B lipid complex“; ABCD) spolu s lokální léčbou konvenčním amfotericinem B a chirurgickou resekcí reziduálního aspergilomu (příloha č. 14). Dívka toto onemocnění, fatální až v 80 % případů, zvládla a stejně tak úspěšně ukončila léčbu základního onemocnění. Kumulativní dávka ABCD byla 2,3 g/kg a nevedla k nefrotoxicitě, což je možné považovat za úspěch adekvátní hydratační léčby s pečlivou korekcí ionogramu, především hypokalemií.

Druhým případem je sterkorální kandidová peritonitida u chlapce s nehodgkinským lymfomem, opět během indukční léčby (příloha č. 15). S obtížemi léčená mykóza byla zvládnuta s konkomitantním podáním výzkumné terapie anti HSP-90 protilátkou efungumab v rámci firemního programu. Chlapec svou mykózu zvládnul a je vyléčen i ze své malignity. Publikované práce s efungumabem referují v den 10 terapie kandózy v 84 % případů zlepšení příznaků s přidáním efungumabu, versus 48 % u pacientů s monoterapií amfotericinem B bez efungumabu, stejně tak byla menší i mortalita den 3 s přidaným efungumabem 4 %, verus 18 % bez efungumabu. Látka efungmab nakonec nesplnila kritéria schválení tržní autorizace pro obavy z fluktuace krevního tlaku a syndromu uvolnění cytokinů a není k použití schválena regulátory.

2.3 Souhrn

Participace v multicentrických a mezinárodních projektech diagnostiky a léčby invazivních mykóz je pro pacienty přínosem v podobě použití standardizovaného postupu diagnostiky a léčby. V případě selhání standardní léčby je použití inovativního postupu plně indikováno. Vytvoření jednotných postupů léčby u imunokompromitovaných pacientů ve formě doporučených postupů je přínosem i pro spolupracující obory.

3 Léčba sarkomů měkkých tkání dětského věku

3.1 Úvod

V dospělé populaci představují sarkomy měkkých tkání (STS) přibližně 1 % všech nádorových onemocnění. U populace dětí a mladých dospělých do 20 let jsou relativně častější a představují 7 % všech nádorových onemocnění, z toho polovina jsou rabdomyosarkomy (RMS). Ostatní nádory této velmi heterogenní skupiny jsou nazývány non-rabdomyosarkomy měkkých tkání (NRSTS, z angl. „non-rhabdomyosarcoma soft tissue sarcomas“).

3.2 Epidemiologie a etiologie

Epidemiologické rozložení STS ve věku do 20 let je velmi pestré. Ve věku do 5 let dominují rabdomyosarkomy s podílem 60 %, naopak mezi 15. a 19. rokem je jejich podíl 23 %. Naopak, NRSTS představují více než 75 % ze všech sarkomů v adolescentním věku. Nejčastějším STS u kojenců je infantilní fibrosarkom, u starších dětí a adolescentů jsou nejčastější synovialosarkom, dermatofibrosarcoma protuberans, maligní nádor pochev periferních nervů (MPNST) a maligní fibrózní histiocytom. Predominance je mírně vyšší u mužského pohlaví v poměru 1,2 : 1. Významným faktorem pro vznik STS je některá z genetických predispozicí, jako Li-Fraumeni syndrom – až 10 % ze všech STS, germinální mutace RB genu. Familiární adenomatosní polypóza je v 25 % případů spojená s agresivní fibromatózou. Neurofibromatóza typu 1 má riziko vzniku MPNST mezi 6 a 13 %. Mutace v SMARCB1 genu je často spojená s extrarenálním maligním rhabdoidním tumorem.

Jako sekundární malignity jsou sarkomy měkkých tkání i kostí známy v dospělé populaci po radioterapii. Při dlouhodobém sledování pacientů léčených pro nádor v dětství bylo zjištěno, že riziko vzniku sarkomu jako sekundární malignity je 9x vyšší než v běžné populaci. Největším rizikem jsou předchozí léčba pro sarkom, anamnéza jiného sekundárního nádoru a radioterapie, nebo vysoké dávkování antracyklinů či alkylancií. Z dalších rizikových faktorů vnějšího prostředí pro vznik angiosarkomu jater je známa předchozí expozice vinylchloridu (u dospělých), chronický lymfedém predisponuje ke vzniku lymfangiosarkomu.

Vyšetření nádorové tkáně, grading, staging

Samozřejmostí při biopsii při podezření na sarkom v dětském věku je uchování biologického materiálu pro další diagnostická vyšetření. Materiál se odesílá na cytogenetické vyšetření, více alikvotů je zamraženo v tekutém dusíku, uchovávají se otisky tkáně pro potřeby FISH analýzy, při dostatečném množství materiálu je odeslán jeho vzorek ke kultivaci buněčných linií. Takto odebraný materiál umožňuje ve složitých případech efektivní diagnostiku pomocí molekulárně genetických metod, které dokáží specifikovat mutace, disrupce a amplifikace genů nebo pomocí RT-PCR detekovat specifické translokace. Staging STS u dětí je tradičně definován chirurgicko-patologickou klasifikací IRS.

Tabulka 1: Staging dětských sarkomů měkkých tkání podle chirurgicko-patologická klasifikace IRS

IRS skupina	Definice
I	Nádor resekován kompletně mikroskopicky (R0)
IIa IIb IIc	Nádor resekován makroskopicky – S mikroskopickým reziduem (R1) a bez postižení lymfatických uzlin Pozitivní lymfatické uzliny kompletně resekovány Pozitivní lymfatické uzliny kompletně resekovány a distální uzlina mikroskopicky pozitivní
III IIIa IIIb	Nádor s makroskopickým reziduem po biopsii (R2) nebo parciální resekci nad 50 % (R2)
IV	Metastázy přítomny, nebo postiženy nadregionální lymfatické uzliny Maligní výpotek nebo implantační metastázy

Diagnóza STS se opírá o probatorní excizi, u menší části pacientů lze provést adekvátní resekci menších a povrchově uložených tumorů. V zásadě nevhodná je tenkojehlová biopsie. Množství materiálu takto odebraného je často limitované, mnohdy nereprezentativní a pro potřeby precizní molekulárně biologické diagnostiky nedostačující.

3.3 Lokální kontrola

Obecný přístup k terapii STS v dětství je podobný jako u dospělých, s některými věkovými specifiky. Tak, jak se liší histologické spektrum STS mezi dospělou a dětskou populací, liší se i přístupy k léčbě. Chování některých STS je v obou populacích podobné, u jiných, jako například u infantilního fibrosarkomu, je diametrálně odlišné. Zda proběhne lokální kontrola chirurgicky, nebo radioterapií, závisí na místě vzniku tumoru a věku pacienta – hůře se dosáhne radikálního záchovného zákroku při menším množství okolních zdravých tkání, a ten může ve výsledku vést k doživotní mutilaci. Pozdní následky radioterapie jsou u dětí mnohem závažnější než u dospělých. Dávky záření efektivní v léčbě STS zastavují další růst ozářených zdravých tkání, které jsou poté hypotrofické.

První důležitým rozhodnutím po diagnóze STS je, jak dosáhnout lokální kontroly. Kdykoliv je to bez mutilace možné, má být provedena chirurgická resekce. Pokud nelze lokální kontroly dosáhnout při akceptovatelné morbiditě, je možné zvážit adjuvantní radioterapii ke snížení rizika lokální recidivy po marginální resekci. Pacientům s primárně neresekabilním tumorem nebo metastázami při diagnóze je nutné nabídnout kombinaci chemoterapie a radioterapie, případně nových léčebných postupů, ať již v rámci paliativní léčby, nebo v současné době rychle se rozvíjejícími možnostmi cílené léčby.

Radikality resekce jako u dospělých není možné zpravidla v končetinových lokalizacích dosáhnout s požadovanou hranicí 2 cm zdravé tkáně. Minimálním požadavkem je odstranění nádorové pseudokapsuly, jinak lze čekat vysokou míru lokálních recidiv, zvláště u STS bez prokázané chemosenzitivity. Naopak u chemosenzitivních STS je marginální resekce přípustná, přežití je u radikálně a neradikálně resekovaných STS srovnatelné.

Postižení lymfatických uzlin není obecně u NRSTS časté, na rozdíl od rabdomyosarkomů. U vybraných vysoce maligních nádorů jako synovialosarkom, angiosarkom, světlobuněčný sarkom nebo extraskeletální Ewingův sarkom může být postižení lymfatických uzlin až v 15 %

případů. Některá centra doporučují ve sporných případech biopsii sentinelové uzliny. Nález pozitivity vede k indikaci resekce a radioterapie.

Přibližně 20 % pacientů se STS má metastatickou nemoc. Predominantním orgánem metastáz jsou plíce. Pokud je možná resekce všech plicních metastáz, měla by být provedena. Dlouhodobého přežití dosahuje pouze 10 % těchto pacientů, ovšem značně se liší mezi jednotlivými typy STS, rabdomyosarkomy mají lepší prognózu než jiné subtypy.

3.4 Systémová léčba

Systémová léčba patří do léčebného postupu u chemosenzitivní choroby. Blíže je uvedena u jednotlivých histologických podtypů níže.

3.5 Sarkomy měkkých tkání typické pro děti a adolescenty a jejich léčba

3.5.1 Rabdomyosarkom (RMS)

Jde o nejčastější STS u dětí a adolescentů s podílem přes 40 %, incidence je 4,3 případu v populaci 1 milionu do věku 20 let. Téměř dvě třetiny případů jsou diagnostikovány u dětí do 5 let. Vyrůstají prakticky v kterékoliv lokalizaci, i když lze pozorovat seskupení do několika skupin. Například lokalizace hlava a krk je typická pro pacienty do 8 let věku. RMS orbity je zpravidla embryonálního typu s velmi dobrou prognózou. Končetinové nádory jsou typické pro adolescenty, převažují u nich alveolární subtypy. Etiologie je podobná jako u všech STS. V rodinách dětí se sarkomem (dvě třetiny z nich byly RMS) a anamnézou spontánního abortu a úmrtím dítěte do jednoho roku věku byla zjištěna v jedné třetině případů některá z forem Li--Fraumeniho syndromu. V souladu s možnými interakcemi mezi genetickou predispozicí a vlivy vnějšího prostředí je zajímavé pozorování až trojnásobně vyššího rizika RMS pro dítě matky, která rok před jeho narozením užívala marihuanu, totéž platí pro otce dítěte. Užívání kokainu bylo spojeno s pětinásobným rizikem.

Charakteristické pro RMS jsou genetické změny. U 70 % případů alveolárního subtypu (ARMS) nalézáme typickou translokaci t(2;13)(q35;q14) nebo t(1;13)(p36;q14) vedoucí ke vzniku fúzního genu PAX3-FOXO1 nebo PAX7-FOXO1. Produkty těchto fúzních genů spolu se ztrátou funkce lokusu CDKN2 vedou k abnormální aktivaci transkripce vedoucí k finálnímu malignímu fenotypu. Podílí na tom i inhibice drah RB a p53 genů, amplifikace genu MYCN a zvýšená exprese receptorové tyrozinkinázy C-MET. Druhý subtyp RMS nazývaný embryonální (ERMS) má typicky ztrátu heterozygozity lokusu 11p15 se ztrátou maternální alely. Na tomto lokusu je gen IGF-2, který kóduje růstový faktor. Ten se pravděpodobně podílí na patogenezi ERMS. Patognomonická je exprese proteinů z rodiny MyoD, které se nevyskytují v jiných než mezenchymálních liniích určených k myogenní diferenciaci. Mutace v genu MyoD1 je spojená s extrémně špatnou prognózou i u lokalizované nemoci. Časté jsou mutace N-RAS a K-RAS onkogenů, které jsou u RMS věkové závislé. První se vyskytuje u novorozenců, druhá má maximum výskytu kolem 14. roku věku.

Téměř 40 % RMS vyrůstá v oblasti hlavy a krku. Důležitá pro prognózu je tzv. parameningeální lokalizace – baze lební, nosní a paranasální dutiny, fossa pterygopalatina/infratemporalis, nosohlatan a střední ucho. V těchto lokalizacích je vysoké riziko lokální recidivy a je indikována radioterapie. Naopak v orbitě nebo v jiných lokalitách hlavy a krku, tzv. non-parameningeálních, je možné radioterapii vynechat z léčebného postupu bez větších rizik a s celkovým přežitím až 95 % pacientů. Další typickou lokalizací jsou genitálie a vývodné cesty močové – trigonum močového měchýře, děloha nebo pochva u dívek nebo nadvarle a prostata u hochů. Končetinové RMS jsou často spojené s postižením regionálních lymfatických uzlin a mají tendenci růst podél fascií. Postižení trupu je rizikové pro lokální recidivy. Obtížně a pozdě jsou diagnostikovány RMS v tělních dutinách nitrohrudí nebo v pánvi. Rychle rostoucí nádor v řádu týdnů vede k dušnosti a je zachycen na prostém RTG snímku. Pomaleji rostoucí nádor může vyplnit celý hemithorax bez dechové tísně, respiračně kompenzační mechanismy dokáží distress eliminovat, a pacient pak přichází s nádorem v celém hemithoraxu s metastázami na pleuře nebo s maligním výpotkem. Z orgánových lokalizací dosahují RMS žlučového traktu zpravidla intraparenchymatózně v játrech obrovských rozměrů, ale mají výbornou prognózu s dobrou senzitivitou na chemoterapii a bez nutnosti velkých chirurgických výkonů. Důvod této biologické vlastnosti znám není.

Léčba rhabdomyosarkomu

Rabdomyosarkom je chemosenzitivní malignita. Kombinovaná léčba chirurgická, radioterapií a chemoterapií je standardem. Intenzita chemoterapie a výběr cytostatik závisí na klinickém

stádiu. Poměrně složitý systém stagingu počítá s parametry, které stratifikují pacienty s lokoregionálním onemocněním do šesti rizikových skupin, z nichž každá vyžaduje jinou intenzitu terapie. Příznivými faktory jsou věk pod 10 let, velikost nádoru méně než 5 cm, lokalizace jiná než parameningeální, končetinová a trup, histologie jiná než alveolární. U choroby metastatické se také vyskytují faktory, které stratifikují pacienta do skupiny s relativně příznivou přibližně 40% prognózou pro vyléčení. Jsou to věk pacienta do 9 let, s nejvýše jednou metastatickou lokalizací (např. plíce) a bez postižení skeletu nebo kostní dřeně.

Chemoterapeutická schémata jsou postavena na vinkristinu a actinomycinu D samotných u pacientů nízkého rizika. Pacienti středního rizika jsou léčení také vinkristinem s aktinomycinem D a s přidáním alkylancia, v Severní Americe cyklofosfamidem (VAC), v Evropě ifosfamidem (IVA). Role antracyklinu je historicky potvrzena jako efektivní, nicméně nevede ke zlepšení léčebných výsledků, jak bylo prokázáno v randomizované studii u vysoce rizikových rabdomyosarkomů. Vzhledem ke kardiální toxicitě se u lokoregionální choroby nepoužívá. Doxorubicin je vyhrazen pro léčbu metastatické nemoci v kombinaci s cyklofosfamidem a vinkristinem (VDC) nebo jako čtyřkombinace ifosfamid, vinkristin, aktinomycin D, doxorubicin (IVADo). Další efektivní kombinací léčiv používaných v terapii metastatické nemoci jsou ifosfamid a etoposid (IE) a vinkristin s irinotekanem (VI).

Načasování chemoterapie záleží na typu chirurgického výkonu při diagnóze. Téměř polovina pacientů spadá do skupiny s neoadjuvantní léčbou. Chemosenzitivní nádor je jednak s výhodou objemově redukován, průkaz chemosenzitivity je biologicky ověřený benefit adjuvantní léčby v trvání až půl roku. Délka adjuvantní léčby je ve všech případech minimálně 6 cyklů opakovaných po třech týdnech. Recentní výsledky randomizované studie s udržovací terapií vinorelbinem a nízkodávkovaným cyklofosfamidem (denně 25 mg/m^2 tělesného povrchu perorálně) podávanými půl roku prokázaly zlepšení přežití u vysoce rizikových pacientů, s dosud nejlepším pětiletým celkovým přežitím 86,5 %. Pacienti s postižením lymfatických uzlin a pozitivitou fúzního genu PAX3-FOXO1 nebo PAX7-FOXO1 mají prognózu srovnatelnou s metastatickou chorobou s pětiletým přežitím bez události 43 % oproti pacientům s N1 chorobou bez pozitivity fúzního genu. Tito pacienti budou v další studii léčeni pomocí delší udržovací terapie.

Relapsy rabdomyosarkomu jsou léčitelné přibližně u třetiny pacientů. Opět záleží na prognostických faktorech a možnostech lokální léčby. Tam, kde byla při primární diagnóze provedena záchovná operace, je u relapsu indikována ztrátová nebo mutilující, pokud vede

k remisi. Radikalita je oprávněná i u radioterapeutických schémat. Léčebné režimy chemoterapie reflektují předchozí linii. Vedle výše zmíněných režimů je efektivní také kombinace vinorelbinu s blokovým cyklofosfamidem (1,2 g/m^2). Kombinace VI s temodalem (VIT) zlepšuje celkové přežití u refrakterních a relabovaných RMS o přibližně čtyři měsíce (10,3 vs. 14,5). Pacienti s refrakterním nebo časně po léčbě první linie relabujícím RMS a pacienti s metastatickou progresí mají velmi malou šanci na dosažení dlouhodobého přežití a měli by být léčení individualizovaně na základě výsledků analýzy nádorového exomu, transkriptomu a profilu aktivity drah proteinkináz a MAP kináz, metylačního profilu a mutační nálože nádoru.

Vybrané non-rhabdomyosarkomy měkkých tkání u dětí a adolescentů

Všechny níže uvedené nádory mají incidenci splňující kritérium vzácného nebo ultra vzácného onemocnění s prevalencí menší než 1 případ v populaci 2 tisíce, resp. 50 tisíc obyvatel. Léčebný přístup tomu odpovídá, mnohdy neexistují žádné údaje o léčbě srovnatelné s populačními randomizovanými studiemi, jako u četnějších diagnóz. Léčba je postavena na historické empirii a publikovaných souborech pacientů s několika desítkami, vzácně stovkami případů, soubory jsou heterogenní. Společným jmenovatelem jsou genetické abnormity a využití cílených léčiv. I léčebná doporučení je nutno touto optikou vnímat jako dosud nejlepší možné standardy péče, včetně precizní diagnostiky a individualizované léčby. Alespoň částečně dávají pacientům s těmito nádory šanci na delší přežití případně kurativní léčbu multikinázové inhibitory cílené na geneticky podmíněné změny ve fúzních genech nebo receptorových tyrozinkinázách nebo MAP kinázách. Ty, pokud jsou v nádoru potvrzeny, bývají dobře zaměřitelné a vedou k dlouhodobé stabilizaci nebo i remisi onemocnění, jako například u NTRK fúzí v případě infantilního fibrosarkomu nebo ALK nebo PDGFRB mutovaných inflamatorních myofibroblastických tumorech nebo agresivních fibromatózách.

3.5.2 Infantilní fibrosarkom

Vyskytuje se výhradně u pacientů mladších čtyř let, 60 % z nich je diagnostikováno do třetího měsíce života, až polovina vzniká *in utero* a je vrozená.

Pro infantilní fibrosarkom (IFS) je charakteristická translokace t(12;15)(p13;25), kterou vznikne fúzní transkript ETV6-NTRK3 (stejná fúze je u vrozeného nádoru ledviny,

mesoblastického nefromu). Tato fúze ovšem není stoprocentně určující, některé IFS ji nemají. Naopak může v patogenezi hrát roli i jiný mechanizmus. Byly nalezeny zvýšené aktivace receptorových tyrozin kináz PI3-Akt, MAPK a SRC bez uvedené fúze.

Histopatologicky jde o vřetenobuněčný vysoce buněčný nádor s častými mitózami, mohou být přítomny okrsky nekróz, zvýšená vaskularita je v okrscích podobných hemangiopericytomu. Původ je pravděpodobně v primitivní mezenchymální vřetenobuněčné buňce, jde o prekurzorovou buňku fibroblastické/myofibroblastické buněčné linie.

Klinický nález je ve dvou třetinách případů měkkotkáňový tumor na končetině, méně často na trupu, krku nebo na kalvě, vzácné je postižení orgánové. Nádor obvykle roste rychle do relativně velkých rozměrů, dvě třetiny pacientů mají nádor větší než 5 cm. Metastatický potenciál IFS je nepatrný.

Metodou léčby je v případě resekabilních IFS chirurgická resekce, pokud v plánovaném rozsahu nepovede k mutilujícímu výsledku. Akceptovatelná je i marginální resekce bez adjuvantní léčby. Vzhledem k relativní velikosti a lokalizaci nádoru to není vždy možné. Mnohem přijatelnější z hlediska funkčních následků léčby je neoadjuvantní chemoterapie. Největší publikované série pacientů s IFS shodně uvádí velmi dobrou léčebnou odpověď kolem 70 % na kombinaci vinkristin a actinomycin D.

Pouze přibližně čtvrtina pacientů je tímto postupem indikována k primární resekci. Intenzifikace léčby o alkylans cyklofosfamid nebo ifosfamid je zpravidla vynucena při stabilním nebo progredujícím nálezu u neresekabilních nádorů. Radioterapie není vzhledem k věku pacientů indikována. Celkové tříleté přežití dosahuje 93 %.

Recentní práce ukazují, že elektivní inhibitor TRK kináz larotrectinib prokázal aktivitu u pacientů s fúzí některého z genů NTRK 1–3. U pacientů s pokročilým nebo metastatickým a relabovaným nebo refrakterním sarkomem s touto genovou fúzí je objektivní radiologická odpověď dosažitelná monoterapií tímto inhibitorem v 93 % případů, což jako recentní poznatek povede k další eliminaci intenzity chemoterapie, zvláště alkylancií.

3.5.3 Inflamatorní myofibroblastický tumor (IMT)

Popisován je také jako zánětlivý pseudotumor nebo zánětlivý (inflamatorní) fibrosarkom. Jde o intermediární nádor s lokálně agresivním růstem. Věkový vrchol incidence je kolem devíti

let. Může se vyskytnout v měkkých tkáních i orgánech, v plicích i dutině břišní. Typický je nespecifickými příznaky doprovázený indolentní růst buď v hrudníku, nebo v retroperitoneu. Diagnóza je uvedena dramatickým nálezem na zobrazovacích metodách (obr. 1). Až třetina pacientů má paraneoplastické projevy, jako zvýšenou sedimentaci erytrocytů, teploty, trombocytózu, anemii, polyklonální hypergamaglobulinemii.

Přesná patogeneze není známa. Příležitostně se uvádí růst po operaci nebo zranění. Histologicky se skládá z myofibroblastů a zánětlivé infiltrace. Poměr těchto složek je proměnlivý a pohybuje se od obrazu fasciitidy k obrazu podobnému buněčnému fibrohistiocytomu nebo hypocelulárnímu desmoidnímu nádoru. Vzácně může být přítomna i kulatobuněčná komponenta.

Imunohistochemicky je u 50 % případů pozitivní tyrozinkináza ALK. Podkladem pozitivity je přestavba genu ALK s fúzními partnery. Pacienti s takovou přestavbou odpovídají na léčbu ALK inhibitorem crizotinibem. Naopak druhá polovina pacientů, ALK negativních (podle imunohistochemie), má agresivnější průběh nemoci. Recentně bylo zjištěno, že tyto ALK negativní nádory mají ALK fúzi s řadou dalších genů. Výsledkem je konstitutivní aktivace fúzního transkriptu s potenciálem terapeutického efektu jinými inhibitory tyrozinkináz.

Klinický průběh onemocnění je variabilní, od benigního po multifokální nebo infiltrativní nádory s tendencí k relapsům. Velikost nádoru s klinickým chováním nekoreluje. Horší průběh je popisován u nádorů vyrůstajících z mezenteria nebo u aneuploidních a s cytologickými atypiemi.

Léčba IMT není vzhledem k variabilnímu klinickému chování jednotná. Resekce, pokud je možná, je metodou volby. Vzácně jsou popisovány i odpovědi na nesteroidní antiflogistika. U relapsů nebo inoperabilních nádorů je indikována radioterapie nebo chemoterapie širokého spektra, od nízce dávkované po alkylans a/nebo antracyklin obsahující režimy s variabilními léčebnými výsledky.

Nově jsou indikovány tyrozinkinázové inhibitory korelující s fúzním stavem genu ALK a jeho partnery, pokud je přítomna konstitutivní aktivace výsledné tyrozinkinázy citlivé na podaný inhibitor. Pacienti, kteří takto definovaný cíl nemají, a i přes veškeré léčebné úsilí progredují, by měli být léčeni individualizovanými přístupy.

3.5.4 Synovialosarkom

Synovialosarkom je u dětí a adolescentů nejčastějším NRSTS, představuje 7,7 % všech STS. Charakteristická je translokace t(X;18)(q11; Xp11) s výslednou fúzí genu SS18 a SSX1 nebo SSX2 vzácně i SSX4. Synovialosarkom nejčastěji vzniká na dolních končetinách (přibližně 60 %) a na horních končetinách (přibližně 20 %). Jde o chemosenzitivní nádor. Léčbou volby je chirurgie, případně radioterapie u marginálně resekovaných nádorů. Nádory do 5 cm mají výbornou prognózu i bez systémové léčby, pokud jsou kompletně resekovány. Metastazování je v těchto případech vzácné. Pacienti s nádory většími než 5 cm mají benefit ze systémové léčby kombinací doxorubicinu a ifosfamidu. Neoadjuvantní léčba je výhodná i jako *in vivo* debulking před záchovnou operací.

3.5.5 Alveolární sarkom měkkých tkání

Jde o nádor s maximem výskytu ve třetí dekádě života. U dětí má maximum výskytu do pěti let života. Typický je nález nebalancované translokace der(17)t(X;17)(p11;q25) s výsledným vznikem fúzního genu APSL-TFE3, který působí jako aberantní transkripční faktor, aktivující dráhu MET kinázy. Potenciálně terapeuticky významná je role TFE3 v indukci imunosupresivního nádorového mikroprostředí, s možností regulace T-efektorovými a T--regulatorními lymfocyty. Průběh bývá zpravidla indolentní. Nádor je chemorezistentní a léčbou volby je chirurgie, případně s adjuvantní radioterapií. Pacienti s lokalizovanou a resekovanou nemocí přežívají v přibližně 70 % případů. Dlouhodobé přežití pacientů s metastázami je přibližně 10 %. Cílená léčiva jako sunitinib, pazopanib, crizotinib vedou ke stabilizaci choroby u většiny MET pozitivních nádorů. Jednoroční celkové přežití v sérii pacientů léčených pro pokročilou nebo metastatickou nemoc crizotinibem bylo 97,4 %.

3.5.6 Světlobuněčný sarkom

Je popisován u dětí mezi 2. a 20. rokem věku, maximum výskytu je u mladých dospělých. Typická je v 90 % detekovaná translokace t(12;22)(q13;q12) s výsledným fúzním genem EWS--ATF1. Ta vede k aktivaci MET kinázy. Vyrůstá v okolí šlach a aponeuróz, histologický obraz

je podobný kožnímu melanomu. Tendence k šíření do lymfatických uzlin vede k nutnosti sentinelové biopsie. Principy terapie jsou chirurgické s možným podílem chemoterapie jako předoperační léčby. Přežití pacientů léčených MET inhibitorem crizitinibem bylo podobné jako u neselektované skupiny se STS léčených doxorubicinem. Vhodný je tento přístup u MET pozitivních nádorů u pacientů s premorbidní kardiomyopatií.

3.5.7 Desmoplastický kulatobuněčný nádor

Vysoce agresivní nádor vyskytující se v tělních dutinách u adolescentů a mladých dospělých se specifickou translokací t(11;22)(q13;q12) s fúzním genem EWS-WT1. Většina pacientů má nádor v břišní dutině nebo v pánvi, často s infiltrací orgánů nebo implantačními metastázami. Volenou léčebnou metodou je multimodální chemoterapie. Resekce víceložiskových nádorů zpravidla není možná. Chemoterapie vede k prodloužení doby do progrese. I přes intenzivní léčbu je pětileté přežití přibližně 15 %, lepší šance mají děti s extraabdominální chorobou. Pokud je dosaženo remise, je šance na přežití v pěti letech přibližně 57 %.

3.5.8 Extrakraniální maligní rhabdoidní tumor

Jde o vysoce agresivní nádor dětského věku, maximum výskytu je do dvou let. Typická je přestavba SMARCB1 genu. Léčba je multimodální. Tendence k časnému metastazování je vysoká. Systémová léčba chemoterapií je efektivní u resekovatelných lokoregionálních onemocnění, je kombinovaná bloky VDC a cyklofosfamid s karboplatinou a etoposidem (CyCE). Čtyřleté přežití je přibližně 40 % u nemetastatické choroby. Metastázy znamenají velmi špatnou prognózu s dvouletým přežitím 13 %. Rizikový je věk do 1,5 roku.

3.5.9 Epiteloidní sarkom

Ze všech STS u dětí představuje epiteloidní sarkom 2 %. Vyskytuje se s maximem kolem 30. roku života, ale je popsán i u kojenců a starších dětí. Typická je genetická aberace – disrupce SMARCB1 genu (dříve popisovaný jako INI1). Histogeneze je nejasná. Vyskytuje se ve dvou

formách – tzv. distální typ, zpravidla jako kožní nebo podkožní léze, šířící se podél aponeuróz a fascií nebo perineurálně a perivaskulárně; může být zaměněn s granulomatózním procesem jiné etiologie, a tzv. konvenční (proximální) typ se znaky podobnými malignímu rhabdoidnímu tumoru včetně disrupce SMARCB1 genu. Má tendenci metastazovat do regionálních lymfatických uzlin, také do plic. Léčba je chirurgická, s adjuvantní radioterapií v případě marginální resekce. Chemoterapie neprokázala benefit, i když může vést ke stabilizaci nemoci. Kinázové inhibitory dosud neprokázaly reprodukovatelný benefit v léčbě této nemoci. Anekdoticky je uváděn benefit eribulinu s minimem nežádoucích účinků.

3.6 Mezinárodní kooperativní skupina EpSSG

Mezinárodní spolupráce v oblasti léčby sarkomů měkkých tkání dětského věku probíhá od časné éry dětské onkologie. V německy mluvících zemích, Švédsku a Polsku je etablována skupina Coperative Weichteilsarkom Studiengruppe der GPOH (CWS). Úzká spolupráce probíhala od 70. let minulého století mezi partnery z Itálie, Velké Británie a Francie a položila základ kooperativní skupiny The International Society of Paediatric Oncology (SIOP), která dala základ studiím Malignant Mesenchymal Tumors (MMT). Postupnou konvergencí jednotlivých národních skupin z prakticky celé Evropy, Izraele, Brazílie, Argentiny a nově Austrálie, došlo v roce 2003 ke sjednocení diagnostických a léčebných postupů pod nově založenou kooperativní skupinou European Pediatric Soft Tissue Sarcoma Study Group (EpSSG, viz https://www.epssgassociation.it/en/). Německy mluvící země si ponechaly organizaci v rámci CWS.

V rámci EpSSG probíhá spolupráce i v České republice. Od roku 2005 byla provedena dvě rozsáhlá akademická klinická hodnocení. Léčba rabdomyosakromů u dětí – studie EpSSG RMS 2005 a léčba non-rabdomyosarkomů u dětí – EpSSG NRSTS 2005. Z obou těchto studií jsou výstupem dosud nejlepší léčebné výsledky ve srovnání s historickými daty a na to navazující velká publikační aktivita.

Studie EpSSG se z počátku potýkaly s rozsáhlými administrativními problémy, které vyplývaly z nově zavedené regulace klinických hodnocení: Clinical trials – Directive 2001/20/EC. Ta klade velké nároky na zodpovědnosti, pojištění, schvalování a povolování a realizaci klinických studií. Problémem byla do té doby v České republice neexistující infrastruktura, která by uvedenou regulaci uvedla do praxe. Konkrétně v zemích s organizační participací národních

skupin onkologie (v Německu – GPOH, Deutsche Krebshilfe, v Itálii – AIEOP, ve Francii – SIOP, ve skandinávských zemích – SSG nebo ve Spojeném království – Cancer Research UK) byla aplikace uvedené legislativy podpořena možnostmi již zavedených sekretariátů, kontaktů, finanční a právní podpory. V České republice podobná organizační struktura nebyla k dispozici. Studie EpSSG byly v České republice v začátcích podporovány v rámci Nadačního fondu dětské onkologie Krtek, který je personálně spojen přímo s Klinikou dětské onkologie FN Brno a díky kterému se podařilo tato klinická hodnocení úspěšně zorganizovat a provést. V roce 2020 je již podpora pro organizaci těchto typů akademických studií zahrnuta ve vládním programu Ministerstva zdravotnictví České republiky.

Studie EpSSG RMS 2005 byla akademická, multicentrická, mezinárodní, „open label“, prospektivní randomizovaná klinická studie prováděná ve 102 centrech ve 14 zemích (Argentina, Belgie, Brazílie, Česká republika, Francie, Irsko, Itálie, Izrael, Nizozemí, Norsko, Slovinsko, Spojené království, Španělsko, Švýcarsko). Obsahovala dvě randomizační otázky. První byla role doxorubicinu u vysoce rizikových pacientů. Ve studii nebyl prokázán vliv na přežití bez události a na celkové přežití pro tuto skupinu pacientů. Tím se doxorubicin vyřadil z léčby lokoregionálních rabdoymyosarkomů a dále není v této indikaci používán. V Evropě zůstává součástí terapeutických schémat pro metastatické rabdomyosarkomy a některé non-rabdomyosarkomy. Druhou otázkou byla role udržovací chemoterapie.

3.7 Komentář k publikovaným pracím

3.7.1 Udržovací terapie u rabdomyosarkomu

V randomizované části RMS 2005 byli do ramene s udržovací chemoterapií zařazeni pacienti, kteří dosáhli indukční léčbou kompletní remise, a jejich vstupní charakteristiky byly nemetastatický rabdomyosarkom buď alveolární histologie bez postižení lymfatických uzlin, nebo embryonální histologie po nekompletní resekci v nepříznivé lokalizaci a věku nad 10 let nebo velikosti tumoru 5 cm, nebo embryonální rabdomyosarkom s uzlinovým postižením (příloha č. 16).

V období 20. 4. 2006 až 20. 12. 2016 bylo screenováno 670 pacientů, z nichž 299 nesplnilo vstupní kritéria zařazení do randomizované části studie. Celkově byl randomizováno 371

pacientů, z nichž 185 léčbu ukončilo v kompletní remisi po intenzivní chemoterapii sestávající z devíti MTD bloků, a u 182 léčba pokračovala ve formě nízkodávkované chemoterapie cyklofosfamid p.o. a vinorelbin i.v. Kritéria modifikace léčby obsahovala podmínky redukce léčiv tak, aby nedocházelo k neutropeniím s absolutním počtem neutrofilů pod 1 000/µl. I přes takto nastavená kritéria byla teplota v neutropenii zaznamenána ve 24 % případů, non-neutropenická infekce v 5 % případů. Po těchto epizodách byla chemoterapie redukována tak, aby nezpůsobovala další neutropenii, raději, než aby byla zcela vysazena. Medián sledování pacientů byl v době analýzy 60,3 měsíců. Výsledky analýzy přežití ukázaly, že pacient s udržovací chemoterapií měli přežití bez onemocnění 77,6 %, pacienti bez udržovací chemoterapie 69,8 % (HR 0,68, p = 0,061), celkové pětileté přežití bylo 86,5 % pro pacienty s udržovací chemoterapií a 73,7 % bez udržovací chemoterapie (HR 0,52, p = 0,0097).

Toto zjištění je po třech dekádách kooperativních projektů v oblasti dětské onkologie u solidních nádorů poprvé, kdy byl prokázán přínos pro celkové přežití u nového chemoterapeutického schématu. Je možné, že udržovací chemoterapie má vliv na přežití i v případě, že pacient bude mít v budoucnu relaps rabdomyosarkomu. V analyzované populaci byl u pacientů s relapsem po udržovací terapii zjištěn relaps o 3 měsíce později než u pacientů bez udržovací chemoterapie. Mechanismem účinku této udržovací chemoterapie by mohl být antiangiogenní a imunomodulační efekt, což by mohlo vysvětlit, proč je relaps zaznamenán později u těch pacientů, kteří udržovací chemterapii měli. Pozorovaným efektem je v této studii menší riziko lokoregionálních relapsů než metastatických. Lokoregionální relapsy jsou hlavní příčinou selhání léčby a mortality. Udržovací chemoterapie je významným prvkem léčby u dětských akutních lymfoblastických leukémií. U solidních tumorů je tato studie první, která prokázala její efekt. Dávka cyklofosfamidu je relativně nízká, 25 mg/m^2 tělesného povrchu. I přesto je nutné další sledování pacientů k vyloučení pozdní toxicity, především ve formě poškození gonád, známého u blokově podávaného cyklofosfamidu a ifosfamidu a sekundárních malignit.

3.7.2 Prognostický vliv genové fúze u alveolárních rabdomyosarkomů s postižením regionálních lymfatických uzlin

Součástí vyšetření nádorové tkáně bylo v randomizované části studie EpSSG RMS2005 i vyšetření stavu fúzních genů typických pro alveolární rabdomyosarkom (ARMS) PAX3-

-FOXO1 nebo PAX7-FOXO1 a určení jejich prognostické hodnoty (příloha č. 17). U 70 % alveolárních rabdomyosarkomů je jedna z těchto fúzí přítomna. Předpokládá se její horší prognostický vliv na přežití.

Cílem této části studie bylo zjistit, zda má stav fúzních genů vliv na parametry přežití při prospektivním sledování. Léčba sestávala z podání 9 bloků MTD chemoterapie ifosfamid/vinkrisitn/actinomycin D a v prvních čtyřech blocích byla intenzifikována o doxorubicin. Lokoregionální léčba byla podána po čtvrtém bloku chemoterapie a sestávala z odložené chirurgické resekce a radioterapie jak na místo primárního nádoru, tak na postižené lymfatické uzliny, bez ohledu na radikalitu resekce (s výjimkou ztrátových končetinových výkonů). Následovala udržovací chemoterapie vinorelbin a nízkodávkovaný cyklofosfamid. Molekulárně biologická analýza byla prováděna v každé z participujících zemí. Jako fúze pozitivní byly označeny nádory s FISH nebo RT-PCR prokázanou pozitivitou v PAX3-FOXO1 nebo PAX7-FOXO1.

Do kohorty ARMS/N1 bylo zařazeno 103 pacientů, z nichž u 85 byla analýza provedena. FOXO1 disrupce byla detekována u 56 pacientů, u 28 byla negativní a u 1 pacienta byl vzorek neadekvátní pro analýzu. Medián sledování pacientů byl 64,9 měsíců. Pětileté přežití bez události bylo lepší ve skupině pacientů bez přítomnosti disrupce FOXO1 genu, 74,4 %, u pacientů s pozitivní disrupcí FOXO1 genu bylo 43 %. Pětileté celkové přežití bylo v těchto skupinách 74,7 % a 43,5 %. Při multivariantní analýze je přítomnost FOXO1 disrupce negativním prognostických faktorem. V univariantní analýze byly jako nepříznivé prognostické faktory identifikovány nepříznivá lokalizace primárního nádoru (tj. jiná než orbita, neparameningeální hlava a krk, vagína, uterus, paratestikulární), invazivita primárního nádoru (T2), přítomnost FOXO1 translokace a klinické stádium IRS III. Z analýzy přežití vyplývá, že pacienti, kteří progredovali po léčbě první linie, měli šanci na přežití pouze 5 %, a proto je legitimní těmto pacientům nabízet inovativní nebo experimentální léčbu prakticky ihned v době relapsu. Lokoregionální relapsy byly zaznamenány ve 42 % případů všech událostí. Lokoregionální staging by měl zahrnovat i „vmezeřené“ uzlinové oblasti, ideálně vyšetřené pomocí FDG-PET, tak aby v případě pozitivit mohl být proveden odběr k vyšetření i těchto uzlin (např. na předloktí, a nejen v kubitě v případě nádoru na ruce) a tím aplikována radioterapie na celou postiženou skupinu lymfatických uzlin.

Pro další generaci diagnostických postupů je doporučeno vyšetření i na jiné fúzní partnery u genu PAX3 pomocí FISH ke zjištění jeho disrupce. Praktickým výstupem této studie je

budoucí zařazení pacientů s ARMS a N1 postižením do stejně rizikové skupiny jako metastatické rabdomyosarkomy, a tím intenzifikovat léčbu s šancí na zlepšení přežití.

3.7.3 Strategie léčby infantilních fibrosarkomů.

Jedním z témat v léčebném protokolu EpSSG NRSTS 2005 byla strategie léčby infantilních fibrosarkomů (příloha č. 18). Tyto nádory se vyskytují v časném věku, jde o nejčastěji se vyskytující nádor měkkých tkání ve věku do jednoho roku, charakteristická je u nich translokace ETV6-NTRK3, vyskytující se u většiny případů. Cílem projektu bylo prospektivně vyhodnotit konzervativní strategii léčby.

Od října 2005 do června 2012 byli do studie prospektivně zařazení pacienti z EpSSG center, staging byl proveden podle TNM a IRS klasifikace. Zařazení byli pacienti s nálezem ETV6--NTRK3 fúze nebo s negativní fúzí, ale konfirmovanou histopatologickou diagnózou s centrálním mezinárodním čtením.

Léčba byla doporučena chirurgickou resekcí v případě, že bylo možné očekávat čisté resekční okraje a chirurgický zákrok nevedl k mutilaci nebo kosmeticky nepřijatelnému efektu. Pokud byl pooperační výsledek IRS I nebo II (R0 nebo R1 resekce), byli pacienti dále sledováni. Neoadjuvatní chemoterapie byla podána pacientům jako vinkristin a aktinomycin D, kromě pacient mladších 3 měsíců, u kterých byla zvolena strategie „wait and watch“, protože i spontánní regrese jsou možné.

Do studie bylo zařazeno 50 pacientů, z toho 19 pacientů ve skupině IRS I-II a 31 pacientů ve skupině IRS III. Chemoterapie byla podána 27 pacientům s mediánem trvání léčby 4,14 měsíce, u 4 pacientů byla zvolena vyčkávací strategie. Cekově byla provedena resekce u 40 pacientů (80 %), z toho 19 jich mělo resekci samotnou, 21 s chemoterapií, tři výkony byly mutilující. V době analýzy bylo 35 pacientů v první kompletní remisi, 7 pacientů ve druhé kompletní remisi, 2 pacienti s reziduálním nálezem, 3 zemřeli a 3 byli ztraceni pro další sledování. Jedno z úmrtí bylo zapříčiněno 100násobným předávkováním aktinomycinu D. Celková odpověď na chemoterapii byla 62,9 %. Adherence k protokolárním doporučením byla velmi dobrá, protože 94,7 % pacientů ve skupině IRS I-II bylo iniciálně léčeno operačním zákrokem a 93,3 % dostalo chemoterapii vinkristin a aktinomycin D. Tím bylo prakticky dosaženo standardizace terapie infantilních fibrosarkomů v centrech napříč Evropou. Diagnosticky byla fúze ETV6-NTRK3

vyšetřena u 87,2 % pacientů. Dříve v některých případech v první linii používané chemoterapeutické režimy s alkylanciem nebo antracyklinem mohou být na základě této studie opuštěny, což vede k významně menší zátěži pacientů s ohledem na akutní i pozdní následky léčby.

3.7.4 Léčba maligních rhabdoidních tumorů

Dalším projektem v rámci EpSSG NRSTS 2005 protokolu byla léčby maligních rhabdoidních tumorů (příloha č. 19). Extrakraniální maligní rhabdoidní tumory jsou velmi agresivní malignita, s nízkou incidencí 0,6 případů na milion dětí, letalita je vysoká s přežitím do 33 % a poslední dekády nedošlo k jakémukoliv zlepšení ve výsledcích přežití. Do studie byli zařazeni pacienti, kteří měli histopatologickou diagnózu podpořenou buď imunohistochemicky negativitou barvení INI1 nebo delecí SMARCB1 genu. Plán léčby byla 30týdenní intenzivní chemoterapie spolu s radioterapií na místo primárního tumoru nebo metastáz, bez ohledu na chirurgickou radikalitu.

Od prosince 2005 do června 2014 bylo do studie zařazeno 110 pacientů, z nich 10 nebylo dále analyzováno pro non-adherenci doporučeného postupu, buď diagnostického, nebo terapeutického. Medián věku byl 1,4 roku, většina ze 77 pacientů měla lokoregionální nemoc, 13 pacientů mělo nádor vrozený (diagnostikován do 4 týdnů věku). Kompletní resekce v první době byla provedena u pouze 8 pacientů, 54 pacientů nedostalo radioterapii, z toho 39 progredovalo dříve, než dospěli k doporučenému termínu radioterapie, a 15 z důvodu rozhodnutí lékaře pro velmi nízký věk (do 1 roku). Medián sledování pacientů byl 44,6 měsíců.

Pro celou kohortu bylo zjištěno tříleté přežití bez události 32,3 % a celkové přežití 38,4 %. Pro pacienty s lokoregionální nemocí bylo čtyřleté celkové přežití 40,1 %, pro metastatickou chorobu bylo dvouleté celkové přežití 13 %. Pacienti se stagingem IRS II měli identifikován jako významný prognostický faktor pro přežití dosažení kompletní remise s čtyřletým celkovým přežitím 66,3 %. Významně horší přežití měli pacienti diagnostikovaní do jednoho roku života, jejich čtyřleté přežití bylo 21,1 %. Iniciální chirurgická resekce nebyla spojena s výhodou pro přežití oproti pacientům s dosažením chirurgické remise později během léčby.

Ve srovnání s dříve publikovanými sériemi bylo v EpSSG skupině více pacientů s extrarenálními nádory, považovanými za prognosticky horší. I přesto bylo dosaženo lepších než dříve publikovaných výsledků přežití.

V současné době je k dispozici cílené léčivo tazemetostat, který je efektivním inhibitorem EZH2 metyltransferázy. Ta je významnou katalytickou podjednotkou komplexu PRC2, mediátoru trimetylace H3K27, který je významný hráčem v onkogenní transformaci. V případě SMARCB1 a SMARCA4 mutovaných nádorů dochází v chromatin remodelujícím komplexu SWI/SWF, který je epigenetickým tumor supresorovým komplexem, k vychýlení rovnováhy diferenciace a proliferace buňky, což vede k tumorigenezi mediované komplexem PCR2. Inhibice podjednotky EZH2 tazemetostatem vede k navození původně vychýleného rovnovážného stavu mezi diferenciací a proliferací. Klinická aktivita tazemetostatu byla potvrzena u celého spektra nádorů, u nichž je EZH2 aktivita zvýšena – nehodgkinských lymfomů, synoviálního sarkomu, mesotheliomu a INI1 negativních nádorů. Klinické použití je od léta 2020 schváleno FDA pro pacienty s epiteloidním sarkomem a folikulárním lymfomem s mutací v EZH2 genu. Pro dětské pacienty je možné podání v rámci firemního „early access“ programu, do kterého jsou na KDO FN Brno zařazení pacienti s INI1 negativními nádory bez možnosti prioritní léčby.

3.8 Souhrn

Léčba sarkomů měkkých tkání dětí a adolescentů je komplexí a vyžaduje multidisciplinární přístup. Nové poznatky z randomizovaných prospektivních studií, na kterých se pracoviště KDO LF MU a FN Brno podílí, jsou příkladem, že zařazení pacienta do multicentrické studie vede k lepším léčebným výsledkům při adekvátní adherenci k doporučeným postupům a generuje nové léčebné postupy rychle uváděné do klinické praxe. Spolu s inovativními léčebnými postupy a přístupem k firemním programům a klinickým hodnocením může pacientovi nabídnout maximum možného v případě refrakterních a relabujících nádorů, a to s reálnou vyhlídkou na léčebný úspěch.

4 Seznam zkratek

ABCD	amphotericin B lipid complex; lipidový komplex amfotericinu B
ADCC	antibody-dependent cell-mediated cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
AML	akutní myeloidní leukemie
ARMS	alveolar rhabdomyosarcoma;alveolární rabdomyosarkom
CAR	chimeric antigen receptor; chimerický antigenní receptor
CELL	Czech Leukemia Study Group for Life; Česká leukemická skupina - pro život
COMBAT	combined oral maintenance biodifferentiating and antiangiogenic therapy; kombinovaná perorální udržovací biodiferenciační a antiangiogenní terapie
COX-2	cyklooxygenáza -2
CML	chronická myeloidní leukémie
CMV	cytomegalovirus
CRISPR	clustered regularly interspaced short palindromic repeats
HDACs	histone deacetylase inhibitors
HPV	human papiloma virus; lidský papilomavirus
FDA	food and drug administration
FOP	fibrodysplasia ossificans progressiva
GIST	gastrointestinal stromal tumor; gastrointestinální stromální nádor
IA	invazivní aspergilóza
IC	invazivní kandidóza
IFS	infantilní fibrosarkom
IMT	inflamatorní myofibroblastický tumor
ITCC	innovative therapies in children with cancer

IRS	intergroup rhabdomyosarcoma studies
KH	klinické hodnocení
MDSC	myeloid-derived supressor cells
MPNST	malignant peripheral nerve sheet tumour
MTD	maximum tolerated dose; maximálně tolerovaná dávka
NRSTS	non-rhabdomyosarcoma of soft tissue; non-rabdomyosarkom měkkých tkání
RMS	rhabdomyosarcoma; rabdomyosarkom
STS	soft tissue sarcoma; sarkom měkkých tkání
TKI	tyrozinkinázový inhibitor
TMB-H	tumour mutation burden-high; vysoká mutační nálož

5 Seznam příloh

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Dendritic Cell-Based Immunotherapy in Advanced Sarcoma and Neuroblastoma Pediatric Patients: Anti-cancer Treatment Preceding Monocyte Harvest Impairs the Immunostimulatory and Antigen-Presenting Behavior of DCs and Manufacturing Process Outcome

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Despite efforts to develop novel treatment strategies, refractory and relapsing sarcoma, and high-risk neuroblastoma continue to have poor prognoses and limited overall survival. Monocyte-derived dendritic cell (DC)-based anti-cancer immunotherapy represents a promising treatment modality in these neoplasias. A DC-based anti-cancer vaccine was evaluated for safety in an academic phase-I/II clinical trial for children, adolescents, and young adults with progressive, recurrent, or primarily metastatic high-risk tumors, mainly sarcomas and neuroblastomas. The DC vaccine was loaded with self-tumor antigens obtained from patient tumor tissue. DC vaccine quality was assessed in terms of DC yield, viability, immunophenotype, production of IL-12 and IL-10, and stimulation of allogenic donor T-cells and autologous T-cells in allo-MLR and auto-MLR, respectively. Here, we show that the outcome of the manufacture of DC-based vaccine is highly variable in terms of both DC yield and DC immunostimulatory properties. In 30% of cases, manufacturing resulted in a product that failed to meet medicinal product specifications and therefore was not released for administration to a patient. Focusing on the isolation of monocytes and the pharmacotherapy preceding monocyte harvest, we show that isolation of monocytes by elutriation is not superior to adherence on plastic in terms of DC yield, viability, or immunostimulatory capacity. Trial patients having undergone monocyte-interfering pharmacotherapy prior to monocyte harvest was associated with an impaired DC-based immunotherapy product outcome.

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Certain combinations of anti-cancer treatment resulted in a similar pattern of inadequate DC parameters, namely, a combination of temozolomide with irinotecan was associated with DCs showing poor maturation and decreased immunostimulatory features, and a combination of pazopanib, topotecan, and MTD-based cyclophosphamide was associated with poor monocyte differentiation and decreased DC immunostimulatory parameters. Searching for a surrogate marker predicting an adverse outcome of DC manufacture in the peripheral blood complete blood count prior to monocyte harvest, we observed an association between an increased number of immature granulocytes in peripheral blood and decreased potency of the DC-based product as quantified by allo-MLR. We conclude that the DC-manufacturing yield and the immunostimulatory quality of anti-cancer DC-based vaccines generated from the monocytes of patients were not influenced by the monocyte isolation modality but were detrimentally affected by the specific combination of anti-cancer agents used prior to monocyte harvest.

Keywords: dendritic cells, anti-cancer medications, sarcoma, neuroblastoma, cell-based medicinal products, investigator-initiated clinical trial, manufacturing outcome variability

INTRODUCTION

Several progressive and relapsing malignancies in pediatric patients have dismal life prognosis. Refractory neuroblastoma and refractory or metastatic sarcoma have an especially poor prognosis, with no consistently curative treatments available. Oberlin et al. (1) published a meta-analysis of North American and European studies on primary metastatic sarcomas and well-defined risk factors that—where two or more are present at presentation—distribute patients into a subgroup with only a 14% event-free and overall survival probability at 3 years from diagnosis. Patients over 10 years of age with limb primary or "other site" primary tumors with the alveolar subtype of rhabdomyosarcoma, bone marrow or bone involvements, and more than three metastatic sites are defined as having markers for a worse prognosis (1). Similar results were published in a study of relapsed rhabdomyosarsomas, with the prognosis for survival being < 10% at 5 years (2). In high-risk neuroblastoma, survival after relapse is poor, and the usual life expectancy is < 6 months. Based on our experience, patients with neuroblastomas with a high MIBG score after induction therapy have very poor 2-year survival (3). High-risk rhabdomyosarcomas are treated according to several globally accepted protocols with a combination of chemotherapy, surgery, and radiotherapy. Chemotherapy regimens consist of the alkylating agent ifosfamide or cyclophosphamide and vinca alkaloids combined with either etoposide or doxorubicin and actinomycin D. The cytotoxic chemotherapy regimens for relapsed and refractory neuroblastoma typically use a combination of camptothecins, topotecan, and irinotecan with agents such as cyclophosphamide and temozolomide, and achieve objective tumor responses but poor long-term outcomes. For such poor-prognosis patients, treatments with innovative and metronomic therapies (e.g., COMBAT, METRO) (4, 5), cell-based immunotherapies (6, 7), and novel molecularly targeted agents (8) are justified and are also effective in many cases, although their long-term effect has yet to be demonstrated.

DCs are essential antigen-presenting cells for the initiation, maintenance, and regulation of immune response (9). Active cancer immunotherapy directs the immune system to attack tumor cells by targeting tumor-associated antigens. We manufacture a fully personalized monocyte-derived dendritic cell-based vaccine that was evaluated in the investigator-initiated clinical trial "Combined antitumor therapy with *ex vivo* manipulated dendritic cells producing interleukin-12 in children, adolescents, and young adults with progressive, recurrent, or primarily metastatic high-risk tumors" (EudraCT number 2014-003388-39). The primary endpoint of the trial was an assessment of safety by analysis of the frequency of occurrence of AESI (adverse events of special interest). Vaccines that meet quality control (QC) requirements are registered for use and applied intradermally every 2–4 weeks for up to 35 doses.

Dendritic cell-based medical products are mostly manufactured through derivation from monocytes. Autologous monocytes are readily accessible and can be obtained from peripheral blood in sufficient amounts to prepare 10^7-10^8 DCs. Monocytes arise from hematological precursors in bone marrow, with a maturation time of 50–60 h (10), and enter the bloodstream for several days until their recruitment into tissues, where they possess the property to mature into tissue macrophages (11). Specifically, the classical CD14++ CD16– subpopulation representing 80–95% of circulating monocytes has a 1-day lifespan in circulation, the intermediate CD14+ CD16+ subpopulation (2–8% of circulating monocytes) has a 4-day lifespan, and the non-classical CD14+ CD16++ subpopulation (2–11% of circulating monocytes) has a 7-day lifespan in circulation (12–14). Monocyte count and function are influenced by various anti-cancer agents. Nevertheless, the published data on the impact of particular anti-cancer agents on the development and function of monocytes are scarce in comparison with those on hematologic toxicity

toward neutrophils and lymphocytes. As most anti-cancer agents target DNA, they interfere with dividing cells including hematopoetic cells. Also, tyrosine kinase inhibitors (regorafenib, sunitinib, sorafenib) are associated with adverse events including hematological toxicities (15). Regorafenib hematological toxicity has been explained by the TK inhibition of FMS like tyrosine kinase 3 (FLT-3) and stem cell factor (c-KIT ligand), which represent hematopoietic growth receptors (15, 16). Reduction in the circulating monocyte count after sunitinib has been shown (17). Monocytes are also highly sensitive to the methylating agent temozolomide (TMZ) (18, 19). Cisplatin and carboplatin have been shown to alter monocyte differentiation to favor the generation of IL-10-producing M2 macrophages (20).

Various chemotherapeutics affect cell differentiation and the antigen presentation of DCs when treated *in vitro* during the differentiation process (21). Data are lacking on the potential *in vivo* impact of hematotoxic agents on the properties of medicinal products from monocyte-derived DCs. During the manufacture of DC-based anti-cancer immunotherapy under stringent GMP-compliant conditions, we experienced highly variable final product parameters in terms of both DC yield and immunostimulatory properties, and we hypothesized that hematotoxic anti-cancer therapy preceding monocyte harvest may influence the quality of DC-based medicinal products. The issue of the effect of pharmacotherapy on the quality of human monocyte-derived DCs cannot be reliably assessed in mimicked conditions by *in vitro* pretreatment of monocytes by anti-cancer agents. Thus, data addressing this issue can only be gathered retrospectively from real-life clinical conditions, such as our clinical trial, though with a limited number of patients included. Here, the Phase-I/II clinical trial protocol designed for heavily pre-treated cancer patients with heterogenic anti-cancer therapeutic protocols allows us to observe and analyze the effect of pharmacotherapy on the quality and presumably also on the anti-cancer action of *ex vivo*-manufactured DCs.

Therefore, our primary aims were to analyze the impact of (i) cytotoxic and targeted anti-cancer therapy preceding monocyte harvest and (ii) variability in the complete blood count on the quality of DC-based anti-cancer immunotherapy in high-risk sarcoma and neuroblastoma patients, representing the two main diagnoses in the DC clinical trial. A secondary aim was to reveal whether monocyte isolation by elutriation is superior to the isolation of monocytes through their adherence to plastic cultivation flasks.

METHODS

Patients and Clinical Trial

Clinical Trial Eligibility and Allowed Medication

Patient eligibility/inclusion criteria for the clinical trial included being 1–25 years old male/female with histologically confirmed refractory, relapsing, or primarily metastatic high-risk tumors and having a performance status (Karnofsky or Lansky score) $\geq$ 50 and a life expectancy of longer than 10 weeks. Patients had to be clinically eligible for the surgical procedure to harvest tumor tissue for histological verification and tumor antigen extraction. Female patients had to have had a negative pregnancy test. All patients had to have adequate bone marrow, kidney, liver, and heart function, defined as absolute neutrophil count (ANC) $\geq 0.75 \times 10^9$/L, thrombocytes $\geq 75 \times 10^9$/L, hemoglobin 80 g/L, estimated glomerular filtration rate (eGFR) $\geq$ 70 mL/min/1.73 m^2, serum creatinine $\leq$ 1.5-fold the upper limit for the appropriate age, bilirubin $\leq$ 1.5-fold the upper limit for the appropriate age, AST and ALT $\leq$ 2.5-fold the upper limit for the appropriate age, ejection fraction $\geq$ 50%, and fractional shortening $\geq$ 27% as assessed by echocardiography. In the case of bone marrow infiltration, the allowable ANC was $\geq 0.5 \times 10^9$/L and blood platelets 40×10^9/L. In case of liver metastases, AST and ALT had to be $\leq$ 5-fold the upper limit for the appropriate age. The exclusion criteria were as follows: seropositivity to HIV1,2, *Treponema pallidum*, hepatitis B or C, known hypersensitivity to the study medication, autoimmune disease that was not adequately treated, uncontrolled psychiatric disease, or uncontrolled hypertension defined as systolic and diastolic blood pressure over the 95th percentile for the appropriate age and height (patients $\leq$ 17 years old) or $\geq$ 160/90 mmHg or diastolic blood pressure $\geq$ 90 mmHg (patients $\geq$ 17 years old). Patients previously treated with dendritic cells or participating in another clinical trial during the 30 days before enrollment were not eligible to enter this clinical trial.

The allowed medication prior to monocyte harvest (leukapheresis) was as follows: metronomic chemotherapy, immune checkpoint inhibitors, and anti-CD20 antibodies were allowed as concomitant medication for any time before leukapheresis. Monoclonal antibodies (except anti-CD20), high-dose chemotherapy, and high-dose corticoids had to have been withdrawn at least 3 weeks prior to leukapheresis with the exception of corticoid treatment of brain edema, which was allowed. Since November 2017, an amendment has been made to the procedure for monocyte harvest, and tyrosine kinase inhibitors have to be withdrawn according to their half-life: drugs with a short half-life of 3–14 h must be withdrawn at least 2 days before leukapheresis (axitinib, dabrafenib, dasatinib, ibrutinib, idelalisib, nintedanib, ruxolitinib, and trametinib), drugs with a medium half-life of 15–35 h at least 7 days before leukapheresis (alectinib, bosutinib, lapatinib, lenvatinib, nilotinib, osimertinib, pazopanib, ponatinib, regorafenib, and non-TKI everolimus), and drugs with a long half-life of 36–60 h at least 12 days before leukapheresis (afatinib, ceritinib, erlotinib, gefitinib, imatinib, cabozantinib, crizotinib, sorafenib, sunitinib, vemurafenib, and non-TKI temsirolimus). Myelopoietic growth factors have to be withdrawn at least 7 days before leukapheresis/monocyte harvest.

Evaluation of Preceding and Concomitant Therapy

A precise analysis was performed of preceding and/or concomitant therapy 60 days before monocyte harvest for clinical trial subjects with neuroblastoma and sarcoma diagnoses. Data were mined from the clinical trial electronic case report form and the subjects' medical records. We particularly focused on therapeutic agents with a potential impact on the generation of DCs from monocytes and on DC immunostimulatory properties. These agents and the reports on their role in monocyte biology are summarized in **Supplementary Table 1**.

DC Manufacture and Quality Control

Dendritic cell vaccine manufacture encompassed two phases—(i) preparation of tumor lysate as a source of the patient's tumor antigens and (ii) preparation of monocyte-derived DCs and their loading with tumor lysate. Quality control tests evaluated safety (negativity for pathogens), identity (cell immunophenotype), viability, and functions (cytokine production, stimulation of T-cells). The flow and decision tree of the manufacturing process is shown in **Supplementary Figure 1**.

Self-Tumor Antigen Extraction

Tumor lysate was prepared from the tumor tissue obtained from the patient during curative surgery or extended biopsy. In Clean Rooms, necrotic areas and connective tissue were removed from the tumor tissue with a surgical scalpel, keeping the specimen immersed in buffered solution. The remaining tissue was sliced into fragments of about 0.5 mm with a scalpel and forceps and then further crushed with the back of a syringe. Each suspension of tumor fragments and cells in HBSS was lysed through repeated (5 times) freezing in liquid nitrogen and thawing at 37°C. The crude tumor lysate was centrifuged at 450 g/7 min/4°C to remove particulate components. The tumor lysate was released for DC manufacture if the following criteria were met: (i) presence of viable tumor cells reported by a histopathologist, (ii) protein concentration, and (iii) microbiological sterility.

Peripheral Mononuclear Cell Collection

Monocytes were harvested as part of the mononuclear white blood cell (WBC) fraction. Mononuclear cells were collected from the peripheral blood of the patient using the Terumo BCT Spectra Optia Apheresis System. For collection, we used either an intermittent or continuous leukapheresis system. Due to its superior collection efficacy and easier procedure settings, we have preferred the continuous leukapheresis system since April 2018. A citrate dextrose solution, solution A (ACD-A), was used as an anticoagulant. In patients with a body weight of < 20 kg, anticoagulation with heparin was used to prevent citrate toxicity. The requirement for the minimal WBC count was 3×10^9/L before the initiation of leukapheresis. To prevent risk of bleeding or ischemic complications during and after the procedure, hemoglobin of at least 80 g/L and platelets of at least 30×10^9/L were required. In case of a patient with a body weight of < 20 kg, the leukapheresis set was pre-filled with donor erythrocytes. The aim of the leukapheresis was to obtain 60–80 mL of concentrate of mononuclear cells with a content of at least 0.5×10^9 monocytes. Subsequent addition of 5% human albumin to the minimum required volume of 80 mL for further processing was allowed.

DC Manufacture in Clean Rooms

The numbers of WBCs, B-cells and T-cells, monocytes, and granulocytes in the leukapheretic product were evaluated using a hematology analyzer (XT-4000i, Sysmex) and flow cytometer (FC-500, Beckman Coulter) with staining for CD3 (clone UCHT1, Beckman Coulter) and CD19 (clone J3-119, Beckman Coulter). Monocytes for DC manufacture were separated from the leukapheresis product by either elutriation or adherence to a plastic surface. During elutriation (using an Elutra cell separator, Gambro BCT), blood cells were separated on the basis of sedimentation velocity into six fractions, where the last fraction rich in monocytes was used for DC manufacture. Contaminating cells after elutriation were mainly granulocytes with similar sedimentation velocity to monocytes. Five hundred million monocytes adhered for 2–4 h in three 175-cm^2 tissue culture flasks with 35 mL of CellGenix® GMP DC Medium at 37°C/5% CO_2 and were then washed with HBSS and processed further. Monocytes seeded from the elutriation product or attached by plastic adherence were then cultivated in three 175-cm^2 tissue culture flasks with 70 mL of CellGenix® GMP DC medium supplemented with GM-CSF (1000 U/mL, CellGenix®) and IL-4 (320 U/mL, CellGenix®) at 37°C/5% CO_2/6 days. On day 3, a fresh 70 mL of medium supplemented with the same concentration of GM-CSF and IL-4 was added to the culture. On day 6, immature DCs were exposed to autologous tumor lysate antigens (10 μg/mL) with added keyhole limpet haemocyanin (KLH, 1 μg/mL), IL-4 (320 U/mL), and GM-CSF (1000 U/mL) at 37°C/5% CO_2/for 1.5–2 h. Maturation was induced by lipopolysaccharide (200 U/mL) and interferon-γ (50 ng/mL) for an additional 6 h at 37°C/5% CO_2. Finally, cells were collected using accutase (Accutase®, Corning), counted in a Bürker cell chamber and frozen in aliquots of 2×10^6 DCs in 100 μL of freezing medium CryoStor® CS2 at -80°C. All doses of the DC-based investigational medical product (IMP) named "MyDendrix®" were stored at -150°C until administration to the patient.

Quality Control of DC-Based Investigational Medicinal Product

DC characteristics were evaluated as a part of the quality control process of IMP from an aliquot of manufactured DC from each batch. The cryotube with DC was removed from a deep freezing box (-150°C) into a laminar flow box, quickly and gently thawed in hand while avoiding shaking, 1 mL of cold (2–8°C) DC medium (CellGenix® GMP-grade) was slowly added to the thawed DCs, and the DC suspension was transferred into 2 mL of cold DC medium. The DC suspension was handled at room temperature and processed immediately. DCs (8×10^5 cells) were seeded into 1 well of a 6-well culture plate for sensitive adherent cells (Sarstedt, TC Plate 6-well, Cell+, growth area 8.87 cm^2) and cultured in 3 mL of DC medium for 2 days (37°C/5% CO_2) to obtain (i) medium containing cytokines produced by DCs during cultivation and (ii) mature DCs for phenotypic evaluation after 2 days of post-thaw cultivation. A 0.5 mL volume of medium containing DC-produced cytokines was collected after 23–25 h upon DC seeding and was centrifuged (10 min/410 g/4°C), and the supernatant was stored at -25°C for no longer than 30 days prior to analysis. For immunophenotypic evaluation of mature DCs, both detached and adherent DCs were harvested 47–49 h after DC seeding. The culture medium was collected and pooled with DCs harvested by accutase (0.5 mL/well 8.87 cm^2/37°C) and centrifuged (5 min/410 g/20°C). The pellet was resuspended in 800 μL HBSS with 0.25% human albumin (Grifols) and processed immediately for immunophenotypic evaluation. Viability quantification was

performed by propidium iodide (PI) exclusion assay. Briefly, 10^5 DCs were stained with 10 μL of 1% PI in HBSS followed by immediate flow cytometric (Cytomics FC500) analysis of PI-positive events (= non-viable cells). The immunophenotype of DCs was evaluated in post-thaw DCs and in post-cultivation mature DCs. For the detection of each surface molecule, 0.5 × 10^5 DCs were incubated for 20 min in the dark with the following antibodies: CD80-PC7 (clone MAB104, 10 μL), CD83-FITC (clone HB15e, 10 μL), CD86-PE (clone HA5.2B7, 10 μL), CD197-PE (clone G043H7, 10 μL), HLA-DR-PC5 (clone Immu357, 10 μL), CD14-PE (clone RMO52, 10 μL), or isotype controls IgG-PC5 (clone 679.1Mc7, 10 μL), IgG-PC7 (clone 679.1Mc7, 10 μL), IgG2a-FITC (clone 7T4-1F5, 10 μL), or IgG2a-PE (7T4-1F5, 10 μL), all from Beckman Coulter. Flow cytometric analysis was performed using a Cytomics FC500 with CXP software by manual gating on individual parameters, and the discrimination by appropriate isotype control was used to gate and quantify positive events. The concentrations of IL-12 and IL-10 in the DC culture medium were measured by flow cytometric bead assay (BD Biosciences) using internal quality controls (Quantikine® Immunoassay Control Group 1, R&D Systems). Absolute production of IL-12 or IL-10 per 10^6 DC and the IL-12/IL-10 ratio were calculated. The allogenic (allo) and autologous (auto) stimulatory properties of DCs were examined by mixed lymphocyte reaction (MLR). In allo-MLR, the target cells were the peripheral blood mononuclear cells (PBMCs) obtained from pooled buffy coats from healthy donors. In auto-MLR, the target cells were the patient's lymphocytes separated by centrifugation in a density gradient using Histopaque-1077 (SigmaAldrich, density 1,077 g/mL) from the leukapheresis product obtained for DC manufacture. These pre-vaccination lymphocytes were cryopreserved using CryoStor CS5 medium (BioLife solutions) at -150°C and thawed prior to auto-MLR seeding. A sample of 10^7 target lymphocytes were stained with 250 μL 10 μM carboxyfluorescein succidimidyl ester (CFSE, SigmaAldrich) and seeded into a sterile 96-well culture plate (Sarstedt, TC Plate 96-well, Suspension, F) at 10^5 cells/well in 200 μL of complete X-vivo 10 medium (Lonza) containing 5% inactivated human male AB serum (SigmaAldrich) for the following: (i) 10^4 DC/well in 10:1 target:effector MLR, (ii) positive control (PC) with phytohemagglutinin (PHA, SigmaAldrich) at a final concentration of 10 μg/mL, or (iii) negative control (NC) with complete X-vivo medium only. MLR experiments were seeded in triplicate and cultured for 6 days at 37°C/5% CO_2. 2 × 10^4 cells from each well were stained with CD3-PC7 (clone UCHT1, 10 μL/test, Beckmann Coulter) for flow cytometric detection of CFSE fluorescence on CD3+ T cells. Discrimination for dividing cells was set up using NC. T-cell proliferation was calculated as follows: [(average % of dividing T-cells in 10:1 MLR) – (average % of dividing T-cells in NC)] × 100/[(average % of dividing T-cells in PC) – (average % of dividing T-cells in NC)].

Statistical Analysis

The Spearman correlation coefficient with a significance test was used to measure the strength of the relationship between patient CBC prior to leukapheresis, the parameters of the leukapheresis product, the DC yield, and the quality control parameters. Differences in parameter values between groups were assessed by the non-parametric Mann-Whitney or Kruskal-Wallis test. Hierarchical clustering analyses were performed using the complete linkage method with the distance based on the Spearman correlation coefficient. The Spearman correlation distance was used for clustering of batches, and the absolute Spearman correlation distance was used for clustering DC parameters. For clustering analyses, DC parameters were centered and scaled (Z-score of parameters). $P < 0.05$ were considered statistically significant. All statistical analyses were performed with R 3.5.3 software (22).

RESULTS

Clinical Trial Accrual and Course

As of May 2019, 47 subjects were enrolled in the clinical trial, and the manufacturing process of DC-based vaccine was performed in 31 cases. Of these 31, the most common diagnoses were sarcoma, with 19 cases (61%), and high-risk neuroblastoma, with 4 cases (**Table 1**). In this group of 23 patients, we performed analysis of the manufacturing issues presented here. Sarcomas were specifically: seven Ewing sarcomas (36% of sarcoma pts), five (26%) osteosarcoma, two (11%) alveolar rhabdomyosarcoma, two (11%) embryonal rhabdomyosarcoma, and three (16%) synovial sarcoma (**Table 1**). The median enrollment age of the clinical trial was 14 years; 15 years for sarcoma patients and 5 years for neuroblastoma patients (**Table 1**). All 23 study subjects, i.e., 19 with sarcoma and four with neuroblastoma, underwent initial surgery to obtain tumor tissue for the tumor lysate-manufacturing process, and tumor lysates were manufactured without any tumor antigen extraction failure. Monocyte harvest and the subsequent manufacturing of DC-based IMP were performed for all 23 subjects. Out of the 23, 16 DC-based IMPs successfully passed through the manufacturing process and met the quality control criteria for administration to the patients. DC-based IMPs from seven subjects (six sarcoma, one neuroblastoma) were not manufactured or failed to pass quality control due to inadequate immunostimulatory properties (**Table 1**). The basic patient characteristics are described in **Table 1**, and the detailed clinical course is summarized in **Supplementary Table 2**.

Dendritic Cell Manufacturing, Its Yield, and DC Quality Including Immunostimulatory Properties

We achieved DC yields ranging from 0 to 43.6%, with a mean of 17.2% and an s.d. of 12.7% in this specific cohort. A DC yield equal to 0 represented a manufacturing process that was unsuccessful, with all DCs detached from the flasks. The quality control parameters involved microbial sterility and *Mycoplasma* spp. negativity, the viability and phenotype of thawed DCs, the phenotype of thawed DCs after 2-day cultivation, the production of IL-12 and IL-10 during 24-h cultivation of thawed DCs, and 6-day allo-MLR and auto-MLR. All batches of DCs fulfilled the microbiological criteria of QC and the criteria

TABLE 1 | DC-based vaccine-manufacturing outcome, basic patient characteristics, therapy preceding monocyte harvest.

Primary diagnosis	Date of study enrollment/Age in years at study enrollment/Pt No	Treatment line prior to monocyte harvest/Treatment and its duration/Date of monocyte harvest	DC-based vaccine-manufacturing outcome
EWING SARCOMA			
Ewing sarcoma of the mandible	09/2015; 14; KDO-0101	2nd; VCR/Irino + pazopanib, 09/2015–04/2016; 01/2016	Passed QC
Localized Ewing sarcoma of the left femur	02/2016; 12; KDO-0109	3rd; ARST08P1 + sunitinib, 03/2016–06/2016; 03/2016	Did not pass QC
Localized Ewing sarcoma of the left distal humerus	02/2016; 12; KDO-0111	2nd; AEWS1031 + pazopanib, 02/2016–08/2016; 05/2016	Did not pass QC
Localized Ewing sarcoma of the spine C5-Th2, extradural, and intraspinal involvement	08/2016; 24; KDO-0118	2nd; AEWS1031, 08/2016–02/2017, 2 cycles VTC, 2 cycles VCR/Irino; 01/2017	Passed QC
Ewing sarcoma of the pelvis	12/2016; 14; KDO-0121	1st; Euro Ewing 2008, 11/2016–05/2017; 06/2017	Did not pass QC
Ewing sarcoma of the left proximal tibia	12/2016; 15; KDO-0122	2nd; VTC cycles, 01/2017–05/2017; 03/2017	Did not pass QC
Localized Ewing sarcoma of the left tibia	08/2018; 22; KDO-0144	2nd; 2x TMZ/Irino, 08/2018–10/2018; 10/2018	Did not pass QC
OSTEOSARCOMA			
Localized high-grade osteosarcoma of the right distal femur	09/2015; 10; KDO-0102	4th; VCR/Irino + pazopanib; 12/2015	Passed QC
High grade osteoblastic osteosarcoma of the left distal femur	10/2016; 8; KDO-0120	1st; AOST 0331, 10/2016–07/2017; 03/2017	Not manufactured
Localized osteoblastic osteosarcoma of the right proximal tibia	01/2017; 18; KDO-0124	3rd; AOST 1321 + VBL + CPM, 02/2017–10/2017; 3/2017	Passed QC
Localized osteosarcoma of the right proximal femur	02/2018; 25; KDO-0133	2nd; COMBAT III, 04/2018–12/2018; 04/2018	Passed QC
High-grade osteoblastic osteosarcoma of the left distal femur	05/2018; 22; KDO-0139	2nd; AOST0331 – cycle IE 07/2018; 09/2018	Passed QC
ALVEOLAR RHABDOMYOSARCOMA			
Alveolar rhabdomyosarcoma of the right calf	10/2015; 14; KDO-0103	2nd; ARST 0921 + TEM, 11/2015–01/2016; 12/2015	Passed QC
Alveolar rhabomyosarcoma, primum ignotum	10/2016; 12; KDO-0119	1st; ARST08P1 + TEM, 10/2016–05/2018; 04/2017	Passed QC
EMBRYONAL RHABDOMYOSARCOMA			
Embryonal rhabomyosarcoma of the pelvis	09/2017; 18; KDO-0131	1st; EpSSG RMS 2005, 09/2017–06/2018; 01/2017	Passed QC
Localized embryonal rhabomyosarcoma of the pelvis	07/2018; 15; KDO-0143	3rd; - rEECur - Topo/CYC, 08/2018–12/2018; 09/2018	Passed QC

(Continued)

TABLE 1 | Continued

Primary diagnosis	Date of study enrollment/Age in years at study enrollment/Pt No	Treatment line prior to monocyte harvest/Treatment and its duration/Date of monocyte harvest	DC-based vaccine-manufacturing outcome
SYNOVIALOSARCOMA			
Synovial sarcoma of the left thigh	04/2016; 14; KDO-0114	1st followed by COMBAT III 05/2015–12/2016; 12/2016	Passed QC
Localized synovial sarcoma of the neck	04/2018; 17; KDO-0137	2nd; Modified COMBAT III from 04/2018 + pazopanib from 08/2018; 06/2018	Passed QC
Localized synovial sarcoma of the left calf	06/2018; 21; KDO-0141	2nd; COMBAT III modified, 08/2018–02/2019; 10/2018	Passed QC
NEUROBLASTOMA			
Neuroblastoma in the retroperitoneum	04/2016; 12; KDO-0115	2nd; METRO-NB2012, 05/2016–10/2016; 07/2016	Passed QC
High-risk neuroblastoma in the left glandula suprarenalis	02/2018; 4; KDO-0135	1st followed by dinutuximab + retinoic acid, 11/2018–02/2019; 02/2019	Passed QC
Neuroblastoma in the right retroperitoneum	07/2018; 3; KDO-0142	2nd; ANBL 1221 - 3 cycles TMZ/Irino + dinutuximab, 08/2018–11/2018; 08/2018	Did not pass QC
Neuroblastoma in the right glandula suprarenalis	10/2018; 6; KDO-0147	4th; METRO-NB2012, 05/2017–12/2018; 11/2018	Passed QC

CPM, cyclophosphamide; Irino, irinotecan; TEM, temsirolimus; TMZ, temozolomide; Topo, topotecan; VBL, vinblastine; VCR, vincristine; IE, ifosfamide etoposid; VTC, vincristine, topotecan, cyclophosphamide; Pt. No., patient number; QC, quality control. Chemotherapy protocols: AEWS1031 (Ewing sarcoma)—vincristine, doxorubcin, cyclophosphamide, ifosfamide, etoposide; AOST0331 (osteosarcoma)—cisplatin, doxorubicine, methotrexate; AOST1321 (osteosarcoma)—denosumab; ARST0921 (refractory or relapsed rhabdomyosarcoma)—bevacizumab, vinorelbine, cyclophosphamide and temsirolimus; ARST1321 (non-rhabdomyosarcoma soft tissue sarcomas)—ifosfamide, doxorubicin, pazopanib; COMBAT III (metronomic)—celecoxib, etoposide, temozolomide, fenofibrate, ergocalciferol, bevacizumab, vinorelbine, cis-retinoic acid; EpSSG RMS 2005 (rhabdomyosarcoma)—ifosfamide, vincristine, actinomycin, doxorubicin; Euro Ewing (Ewing sarcoma)—vincristine, ifosfamide, doxorubicin, etoposide, actinomycin, cyclophosphamide; METRO-NBL2012 (metronomic treatment for neuroblastoma)—etoposide, celecoxib, propranolol, cyclophosphamide, vinblastine; rEECur protocol (relapsed soft tissue sarcoma)—topotecan, cyclophosphamide, irinotecan, temozolomide. Details on anti-cancer therapy dosing are summarized in ***Supplementary Table 2****.*

of viability, ranging from 85 to 100% with a mean of 95%. Their variability in phenotype and immunostimulatory property is shown in **Supplementary Table 3**. The mean phenotype of the manufactured DCs immediately after thawing for selected parameters was as follows: CD8019 (range: 2–86%), CD86 91% (76–100%), CD83 21% (0–86%), CD14 20% (1–69%), and CD197 90% (73–99%). The mean phenotype of thawed DCs after 2-day cultivation for selected parameters was as follows: CD80 77% (range: 25–97%), CD86 99% (95–100%), CD83 61% (12–89%), and MHC II 93% (63–100%). Mean cytokine production was as follows: IL-12 8,327 pg/10^6 DC (range: 9–80,824 pg/10^6 DC), IL-10 280 pg/10^6 DC (6–1,731 pg/10^6 DC), and IL-12/IL-10 ratio 35 (1–246). The mean *in vitro* proliferation of T-cells stimulated by manufactured DCs was 67% (29–98%) in allo-MLR and 9% (−3–37%) in auto-MLR. Due to inappropriate results for the immunostimulatory parameters of QC (phenotype, cytokine production, MLR), six out of 22 (27%) of the manufactured batches of DCs were not released for use in the clinical trial. The parameter values of the manufactured batches of DCs are shown in **Supplementary Table 3**.

Isolation of Monocytes by Adherence vs. Elutriation and Its Impact on Manufacturing Process Yield and the Immunostimulatory Parameters of DCs

Isolation of monocytes for DC manufacture was performed by elutriation in 14 cases and by plastic adherence in nine (39%) cases based on the real-world situation. Until March 2017, we performed elutriation of the leukapheresis product in all cases (11 cases: KDO-0101, -0102, -0103, -0109, -0111, -0114, -0115, -0118, -0120, -0122, -0124). Between April and September 2018, we performed elutriation in cases KDO-0121, -0137, and -0139, and adherence to plastic in cases KDO-0133, -0142, and -0144 due to there being > 10% neutrophils in the leukapheresis product or technical issues with the Elutra device for KDO-0119 and -0131. After October 2018, we isolated monocytes exclusively by adherence to the plastic surface in all cases: KDO-0135, -0141, -0144, and -0147.

Addressing the issue of whether the elutriation process is superior to adherence to plastic retrospectively, we compared the proportions of batches passing QC and their DC yield and phenotypic and immunostimulatory properties under the

two methods. Adherence to plastic resulted in two (22%) batches not being released, and elutriation resulted in five (36%) batches not being released (four did not pass QC and one was not manufactured). The OR (odds ratio) for passing QC in the plastic-adherence modality was 1.94 (95% CI: 0.29–13.19). The DC yield, viability, phenotype, and immunostimulatory properties (IL-12, IL-10, the IL-12/IL-10 ratio, allo-MLR, auto-MLR) in adherence to plastic vs. elutriation are summarized in **Figure 1**. A statistically significant difference was observed between QC results and monocyte isolation modality for the following post-thaw parameters (i) DC expression of CD86 on day 0 that was higher in the manufacturing process with plastic adherence, and (ii) borderline significant expression of CD14 on day 0 that was higher with elutriation. The values of both parameters were in favor of adherence to plastic. It is of note here that the subgroup with isolation of monocytes by the adherence to plastic was not biased by including a higher proportion of cases without potentially monocyte-interfering pharmacotherapy ("m" vs. "0" as described later; $p = 0.643$). Thus, we conclude that the isolation of monocytes by adherence to plastic is comparable to a manufacturing process with monocyte elutriation.

Parameters of CBC Prior to Monocyte Harvest, and Parameters of the Leukapheresis Product and Their Impact on Manufacturing Process Yield and the Immunostimulatory Properties of DCs

With the aim of identifying the CBC parameters (shown for each batch in **Supplementary Table 3**) associated with adequate DC characteristics and thus predicting whether the DC-manufacturing process would pass QC, we analyzed CBC prior to monocyte harvest in the context of batches that fail to pass QC and DC yield, phenotype, and immunostimulatory properties. The presence of immature granulocytes in CBC was associated with unsuccessful manufacturing ($p = 0.046$). DC yield was not associated with any single parameter of CBC. Expression of CD14 on manufactured cells was negatively correlated with relative lymphocyte count in CBC ($p = 0.001$) (**Figure 2**). The level of allogenic MLR was negatively associated with both the presence of immature granulocytes ($p = 0.010$) and NRBC ($p = 0.018$)

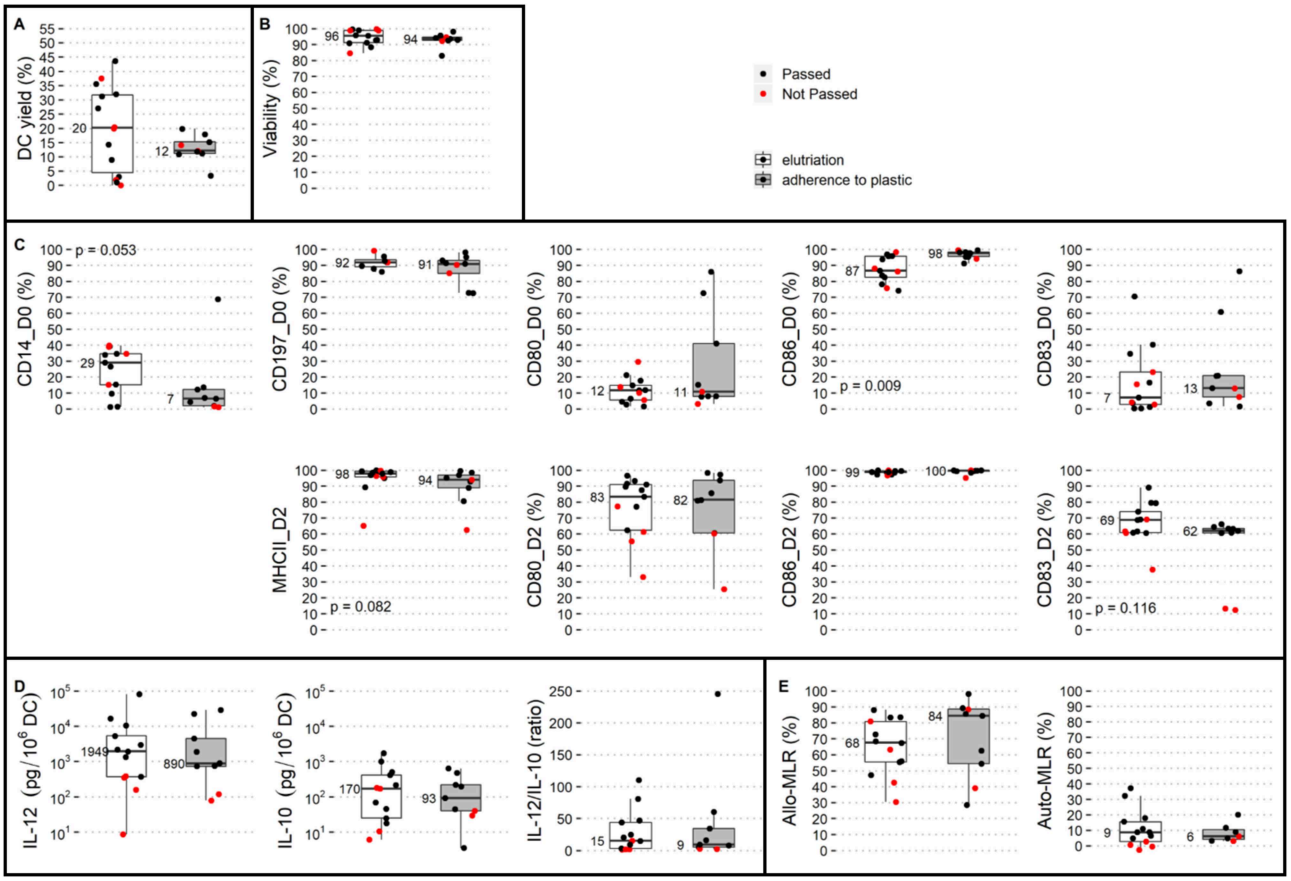

FIGURE 1 | Comparison of two monocyte isolation modalities with respect to dendritic cell (DC) production. Elutriation (white box plots) and adherence to plastic (gray box plots) were compared based on QC parameters: **(A)** DC yield, and post-thaw: **(B)** viability, **(C)** DC phenotype on day 0: CD14, CD197, CD80, CD86, and CD83 and on day 2: MHC II, CD80, CD86, and CD83, and immunostimulatory properties presented by **(D)** IL-12 production, IL-10 production, and IL-12/IL-10 production ratio, **(E)** allo-MLR and auto-MLR. Median values are shown for each parameter for each monocyte isolation modality. Black dots show QC results of manufactured DCs that passed quality control, and red dots show results of manufactured DCs that did not pass quality control.

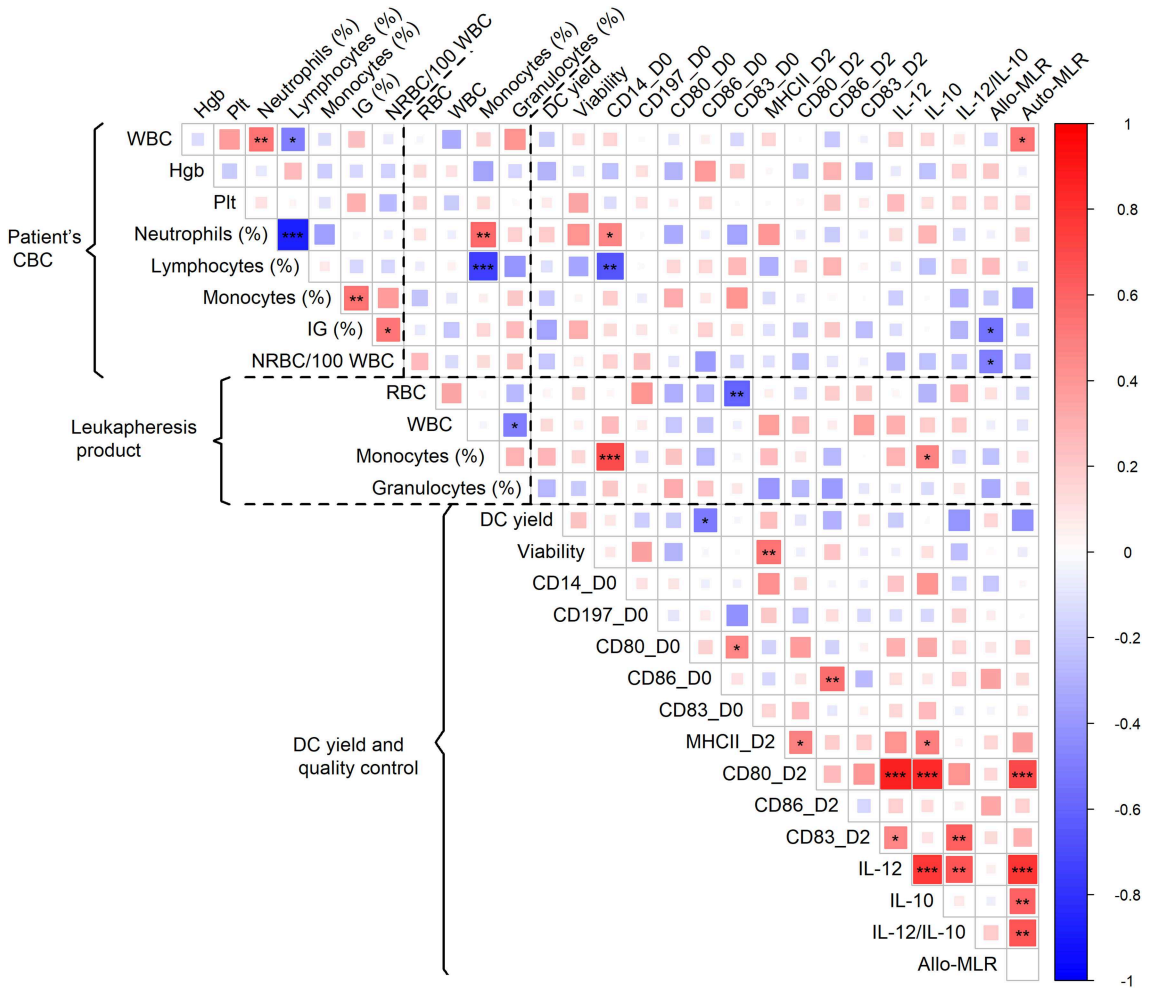

FIGURE 2 | Association of patient CBC prior to monocyte harvest and parameters of leukapheresis product with DC yield and quality control. Red color represents a positive correlation and blue color a negative correlation; strength of relationship is represented by size of square and intensity of color—larger squares with intense color have a stronger association; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

in pre-leukapheresis CBC (**Figure 2**). The level of autologous MLR was positively associated with absolute leukocyte count ($p = 0.016$) (**Figure 2**). Similarly, a high proportion of monocytes ($p < 0.001$) and low proportion of T-cells ($p = 0.001$) in the leukapheresis product were associated with increased expression of CD14 on manufactured cells (**Figure 2**). A high proportion of monocytes in the leukapheresis product was associated with increased production of IL-10 by manufactured cells ($p = 0.027$) (**Figure 2**).

Therapy Preceding and/or Concomitant With Monocyte Harvest and Its Association With Manufacturing Process Yield and the Immunostimulatory Properties of DCs

The patient history of anti-cancer treatment and the outcome of DC manufacture were evaluated for an association between DC parameters and lines of therapy classified as 1st, 2nd, and 3rd or subsequent lines that were followed by monocyte harvest for DCs. The history of anti-cancer treatment had no observed impact on the quality of manufactured DCs (**Supplementary Figure 2**). Pharmacotherapeutics 60 days prior to and/or concomitant to monocyte harvest were classified into two groups and designated as follows (i) "m" ($n = 17$) for administration of therapy potentially interfering with monocyte viability and/or differentiation, namely TKI, mTOR inhibitors, chemotherapy in cell biology-interfering doses, i.e., MTD-based dose, anti-RANKL mAb, retinoic acid, and/or G-CSF (**Supplementary Table 1**) < 60 days prior to monocyte harvest, (ii) "0" ($n = 6$) for metronomic therapy/chemotherapy or no potentially monocyte-interfering therapy concomitantly or < 60 days prior to monocyte harvest. All batches from the "0" category passed QC, whereas seven out of 17 (41%) monocyte-derived DCs from the "m" category failed to be released for patient administration. The OR for passing QC in category "0" was 9.3 (95% CI: 0.5–191). DC yield, DC immunophenotype on day 0 and day 2, and production of IL-10 did not differ between

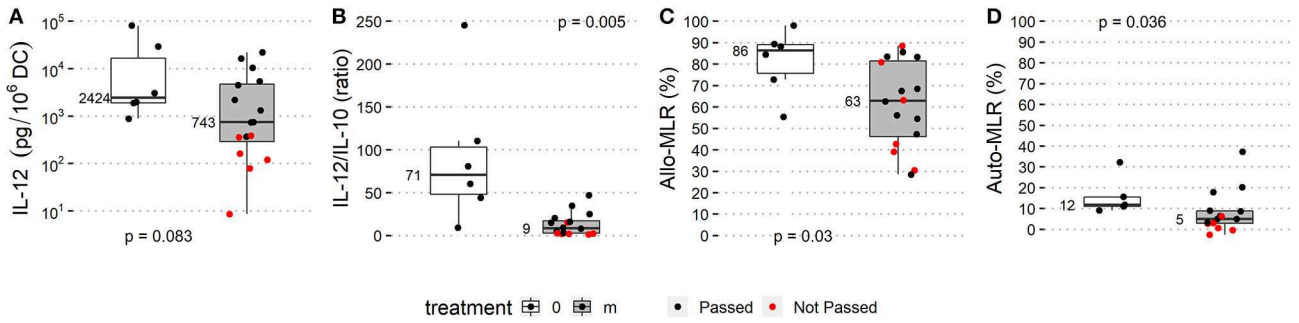

FIGURE 3 | Treatment prior to monocyte harvest and immunostimulatory properties of manufactured DCs. Manufacturing subgroup from monocytes harvested after MTD-based therapy potentially interfering with monocyte biology (listed in **Supplementary Table 1**; "m" treatment, gray box plots) and manufacturing subgroup from monocytes from untreated patients or after non-interfering treatment ("0" treatment, white box plots) were compared based on QC parameters: **(A)** IL-12 production, **(B)** IL-12/IL-10 production ratio, **(C)** allo-MLR and **(D)** auto-MLR. Median values are shown for each parameter for each treatment subgroup. Black dots show QC results of manufactured DCs that passed quality control, and red dots show results of manufactured DCs that did not pass quality control.

the "0" and "m" categories (**Supplementary Figure 3**). Median IL-12 production was 2,424 pg/10^6 DCs in the "0" category and 743 pg/10^6 DCs in category "m" ($p = 0.083$). The median IL-12/IL-10 ratio was 71 in the "0" category and 9 in the "m" category ($p = 0.002$). The median T-cell proliferation in allo-MLR was 86% in the "0" category and 63% in the "m" category ($p = 0.027$), and the in auto-MLR was 12% in the category "0" and 5% in category "m" ($p = 0.036$) (**Figure 3**).

In the analyzed study cohort, therapeutic regimens were heterogenic, with patients often treated with a combination of various compounds prior to monocyte harvest, and thus further categorization into single agent-defined subgroups and their analysis were impossible. Therefore, we performed cluster analysis of DC parameters in the context of therapy prior to monocyte harvest (**Figure 4**). Here we observed a cluster defined mainly by a superior IL-12/IL-10 ratio but low DC yield comprising batches KDO-0133 without any anti-cancer treatment, KDO-0137 treated with metronomic modified COMBAT with celecoxib, fenofibrate, low-dose cyclophosphamide, and low-dose vinblastine, and KDO-0115 treated with metronomic therapy with low-dose vinblastine, celecoxib, low-dose cyclophosphamide, and propranolol (see **Supplementary Table 2** for details on the treatment schedule and dosing). Furthermore, we observed a very similar pattern in DC properties in two batches, KDO-0142 and KDO-0144, that were manufactured from monocytes obtained from patients treated with temozolomide and irinotecan. These batches exhibited robust monocyte differentiation, as represented by their low CD14 expression, but failed to produce IL-12 or an immunostimulatory phenotype when matured, as represented by CD80 on post-cultivation DCs on day 2, and therefore did not meet the QC criteria. A pattern of relatively low DC yield, high production of IL-12, and notable monocyte differentiation and DC immunostimulatory phenotype and function was observed for batches KDO-0147, generated from monocytes from patients treated with celecoxib, and KDO-0141, from patients pretreated with combined metronomic therapy with low-dose vinblastine, low-dose etoposide, celecoxib, cholecalciferol, and fenofibrate. Batches KDO-0103 and KDO-0122 similarly exhibited poor yield, poor monocyte differentiation, a rather low IL-12/IL-10 ratio, and very low immunostimulatory functions toward donor T-cells. Monocytes from both batches were pretreated with an MTD-based combination of topoisomerase inhibitor and alkylating agent, with last administration from day 21 to 17, namely etoposide and ifosfamide in KDO-0103 and topotecan and cyclophosphamide in KDO-0122. This was followed in both cases by 9 days of administration of G-CSF filgrastim up to 7 days prior to monocyte harvest. High DC yield and viability but low markers of differentiation, immunostimulatory phenotype and IL-12/IL-10 ratio were similarly observed for batches KDO-0111 and KDO-0109 treated with topotecan, cyclophosphamide, and pazopanib. Based on features such as good DC yield and viability but low monocyte differentiation and a below-average IL-12/IL-10 ratio, these two batches clustered with KDO-0139 (treated with etoposide, ifosfamide, and filgrastim), KDO-0121 (etoposide, ifosfamide, and filgrastim), KDO-0118 (irinotecan and sunitinib), and KDO-0119 (cyclophosphamide, temsirolimus, and filgrastim). Notably, monocytes affected by retinoic acid (KDO-0135) or anti-RANKL denosumab (KDO-0124) produced DCs of average quality. In summary, monocyte-interfering MTD-based treatment of the clinical trial patients prior to monocyte harvest was associated with an impaired DC-based immunotherapy manufacturing process outcome. Certain combinations of anti-cancer treatments elicited a similar pattern of inadequate DC parameters. Namely, a combination of temozolomide and irinotecan was associated with poor DC maturation and immunostimulatory features, and a combination of pazopanib, topotecan, and MTD-based cyclophosphamide was associated with poor DC differentiation maturation and immunostimulatory parameters.

DISCUSSION

Here we show that despite strict adherence to the validated manufacturing protocol, the outcome of the manufacture of

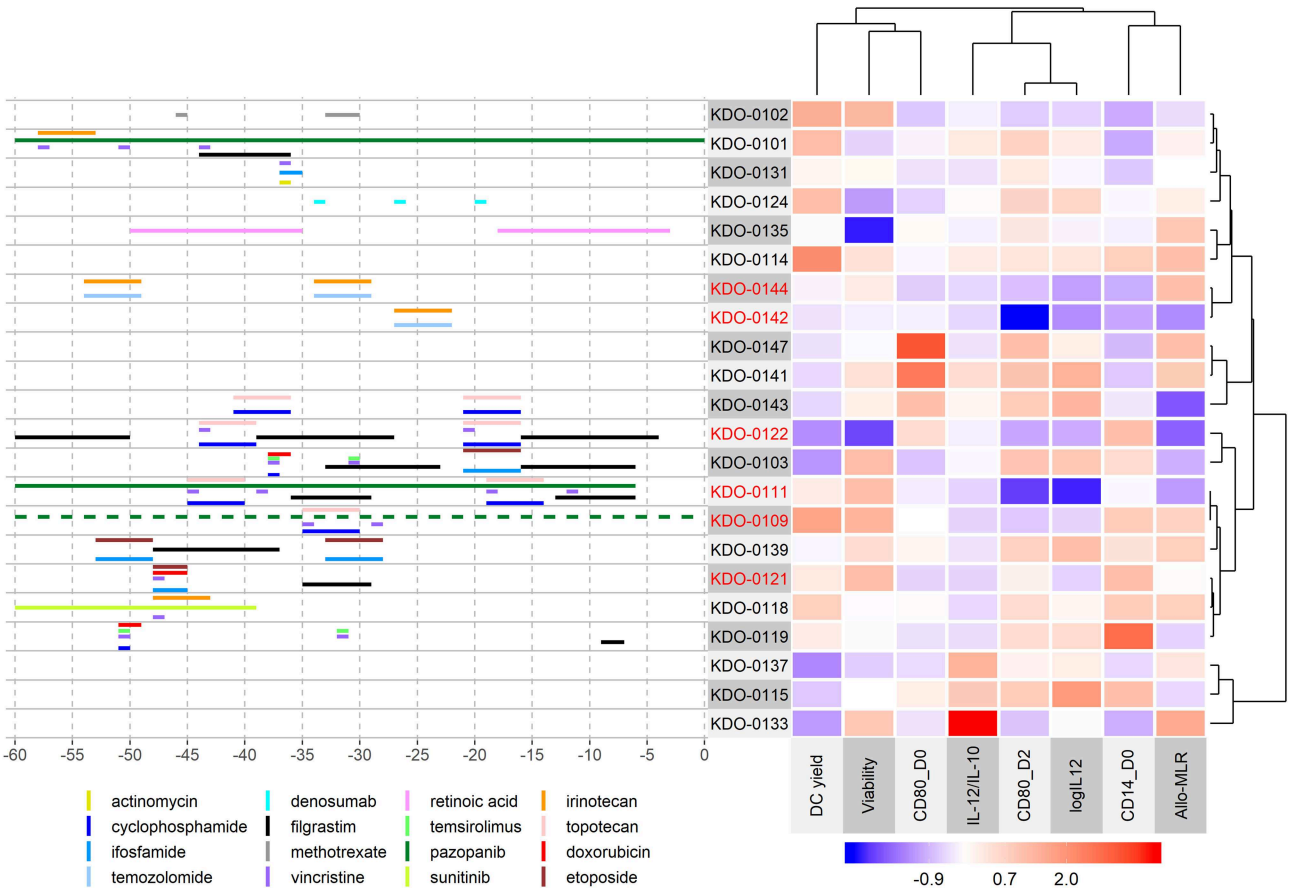

FIGURE 4 | Cluster analysis of DC parameters in the context of therapy prior to monocyte harvest. The heatmap on the right shows the immunostimulatory properties of manufactured DCs centered and scaled in the column direction (Z-score of parameters). Clusters are based on correlations. For clustering of DC parameters, but not batches, an equal meaning to positive and negative correlations was considered, and therefore strongly correlated parameters in the positive or negative manner clustered together. The left panel shows the treatment administered within 60 days of monocyte harvest. The day of the mononuclear harvest was set as day 0. An interactive version of the left panel with a detailed description of treatment including dosing is provided in **Supplementary Material 1**. Metronomic doses of chemotherapeutic drugs and supportive therapy such as vitamins and probiotics are not shown here but are summarized in **Supplementary Table 2**. Batches that did not pass quality control are indicated in red.

the medicinal product with monocyte-derived DCs is highly variable in terms of both DC yield and immunostimulatory properties. Moreover, in 30% of cases, manufacture of DC-based immunotherapy for advanced sarcoma and high-risk neuroblastoma patients resulted in a product that did not meet the specifications for the medicinal product and therefore was not released for application. This product failure rate was higher than in published studies (23, 24). Thus, in an attempt to improve the manufacturing process, to predict DC-manufacturing outcome, and, subsequently, to avoid laborious and costly DC manufacture that would not meet QC specifications, we addressed key variables in the manufacturing process. Namely, we focused on the issues of (i) monocyte isolation from the mononuclear leukapheresis product, (ii) parameters of the patient's CBC prior to monocyte harvest and parameters of the leukapheresis product, and (iii) anti-cancer therapy preceding monocyte harvest that may interfere with the ability of monocytes to differentiate into immunostimulatory DCs.

Regarding the method of monocyte isolation, we assessed whether monocyte extraction by a simple method of adherence to a plastic surface is comparable to the elaborate method of elutriation. During elutriation, monocytes can be contaminated with granulocytes with a similar sedimentation velocity to monocytes. Based on this observation, we validated the DC-manufacturing process with isolation of monocytes by adherence to plastic (25) to avoid contaminants that may interfere with DC differentiation by altering the levels of pro-differentiation cytokines and/or the formation of a suppressing microenvironment through generating decay products during cultivation. By comparative analysis of DC yield and immunostimulatory properties from the manufacturing processes of isolation of monocytes by elutriation vs. adherence to plastic, we conclude that the adherence method is comparable to the elutriation method. The method of adherence to plastic is simple in terms of the equipment, material, and manufacturing steps required and therefore is less costly, less prone to errors,

and more GMP-friendly than the elutriation process. In healthy adult volunteers, monocyte-derived DC yield with monocyte elutriation has been shown to be superior to adherence to plastic (26); this was not observed under our manufacturing conditions of heavily pretreated pediatric sarcoma and neuroblastoma patients.

With regards to the pharmacotherapy preceding monocyte harvest, we observed that therapy with agents interfering with the biology of monocytes 60 days prior to monocyte harvest was associated with reduced production of IL-12 and deficient functional immunostimulatory properties of the manufactured DC-based vaccine and subsequently often resulted in QC failure. It is of note here that failures in DC production occurred more often prior to the implementation of stricter criteria for non-allowed pharmacotherapy preceding monocyte harvest. Specifically, we observed impaired monocyte differentiation and, subsequently, inadequate immunostimulatory features in monocytes pretreated with a combination of an MTD-based dose of the alkylating agent cyclophosphamide, topoisomerase I inhibitor topotecan, and TKI pazopanib. We have previously shown that TKI pazopanib *in vitro* impairs the immunostimulatory properties of monocytes, including up-regulation of the immunoinhibitory surface molecule ILT-3 and decreased capability to up-regulate MHC II in response to LPS (27). Interestingly, however, pretreatment of monocytes *in vivo* with pazopanib without any other immediate treatment (KDO-0101) did not result in attenuated DC vaccine quality. Topotecan has been shown to partially activate monocyte-derived DCs but to prevent the full maturation of DCs stimulated with a cocktail of proinflammatory mediators (28). A different pattern was observed for DCs from cases treated with a combination of the alkylating agent temozolomide (TMZ) and the topoisomerase I inhibitor irinotecan (iri), and we observed monocyte differentiation but not DC immunostimulatory properties, resulting in a medicinal product that did not pass QC and was not administered. It is of note that one case was a sarcoma and one a neuroblastoma patient. Moreover, we also observed a similar pattern of poor DC parameters in a case of synovialosarcoma with TMZ/iri therapy in a cohort of patients outside this clinical trial. It has been shown that monocytes are particularly sensitive to the methylating agent temozolomide, undergoing apoptosis, while monocyte-derived DCs and macrophages are resistant to TMZ (19). Briegert and Kaina and Bauer et al. showed that monocytes accumulated single-strand DNA breaks due to failure of the re-ligation step in base excision repair and showed a lack of DNA repair protein expression (18, 19). Following TMZ treatment, monocytes demonstrated an unbalanced expression of DNA repair proteins, impairing base excision repair and the accumulation of double-stranded breaks (18, 19). *In vitro* studies of TMZ/iri cytotoxicity to neuroblastoma cells have revealed single- or double-stranded DNA damage to be mostly due to SN-38 (the active metabolite of irinotecan) and to be further enhanced through the addition of TMZ (29). Thus, we hypothesize that DNA damage caused by the combination of irinotecan and TMZ in the context of particular hypersensitivity of monocytes to temozolomide may underlie the unfavorable effect of anti-cancer therapy with TMZ/iri on the monocyte-derived immunostimulatory DC-manufacturing process. Monocytes from a patient treated with methotrexate, doxorubicin, and cisplatin failed to produce viable dendritic cells, but monocytes from another patient treated with methotrexate did not fail to produce DC vaccine. Methotrexate has reportedly inducedl apoptosis, reduced viability, induced differentiation, and reduced inflammatory properties of monocytes (30–33), and we may speculate, although based on anecdotal observation, that if combined with cisplatin, thereby shifting monocyte differentiation into an immunosuppressive phenotype (20), methotrexate may result in failure of monocyte-derived DC generation.

Regarding the composition of pre-leukapheresis CBC and the derived leukapheresis product and the outcome of DC manufacture, we observed that three interconnected features, i.e., (i) a low relative lymphocyte count, (ii) a high relative neutrophil count in CBC, and (iii) a high proportion of monocytes in the leukapheresis product, were associated with unfavorably high expression of CD14 on the manufactured cell product. Moreover, the presence of an increased number of immature granulocytes was associated with decreased potency of the DC-based product as quantified by allo-MLR. These observations may be underlain by emergency myelopoesis stimulated by G-CSF, which leads to a quantitative and qualitative change in all circulating myeloid cell types including neutrophils, monocytes, and myeloid-derived suppressor cells (34, 35). While fostering granulocyte effector functions, G-CSF also seems to promote immunosuppressive and tolerogenic properties in monocytes and monocyte-derived cells including increased production of IL-10 (36–39). In this context, it is of note that six out of seven cases treated with G-CSF within 60 days prior to monocyte harvest exhibited donor T-cell stimulation below the average and that the level of T-cell stimulation decreased with the intensity of G-CSF prior to monocyte harvest. Although the effect of G-CSF treatment on the DC-manufacturing process in our study cannot be dissected from the effect of preceding chemotherapy and targeted therapy, the tentative interpretation is that stimulation of myelopoesis with growth factors of granulocytes may have a rather negative impact on the outcome of the DC-based vaccine-manufacturing process.

Here, we show that treatment of patients with certain anti-cancer agents in MTD-based doses prior to monocyte harvest often leads to failure of manufacture of the immunostimulatory DC-based vaccine. We propose that the optimal time for monocyte harvest for generating DCs is prior to a cell-interfering treatment. With respect to the DC-manufacturing workflow, this would mean, in a majority of cancer patients, the implementation of DC manufacture from cryopreserved monocytes. Several studies have investigated the effect of cryopreservation on monocyte differentiation into DCs, but results have been conflicting. Some studies observed cryopreservation to have no effect on monocyte-derived DC production (40, 41). On the other hand, Silveira et al. showed that, when compared to fresh monocytes, cryopreserved monocytes exhibited impaired differentiation into dendritic cells, with lower rates of maturation and cytokine production in response to LPS and lower lymphocyte proliferation in allo-MLR (42). Thus, the cryopreservation of monocytes for

DC generation may decrease the quality of manufactured DCs, and the level of this decrease needs to be specified for a particular manufacturing protocol. In case of a minor drop in DC maturation and immunostimulatory parameters and function due to the cryopreservation of monocytes, this manufacturing modality should be considered, as it would allow harvesting of therapy-naïve monocytes and avoid a potentially detrimental effect of certain anti-cancer and supportive treatment on the quality of DC-based anti-cancer immunotherapy.

Another issue in the context of the concurrence of anti-cancer treatment and monocyte-derived DC manufacture is the length of the pharmacotherapy-free period prior to monocyte harvest. From our real-life experience gained on this study group, we conclude that a 30-day interval without treatment is not sufficient for the combination of temozolomide and irinotecan to sufficiently wash out the monocyte biology-interfering effect of this combination. However, the issue of a safe therapy-free window is not likely to be addressable through the establishment of a wash-out period for a particular drug. The fitness of monocytes and their capacity to differentiate and mature into DCs with high antigen-presenting effect is a matter of their biological function in the context of iatrogenic affection, which is complexly shaped by the need for immediate treatments, their combinations, their cumulative doses, and the long-term history of treatment. Therefore, identifying a marker revealed from a patient's peripheral blood that predicts the outcome of DC-generation would help to avoid an unproductive anti-cancer DC-manufacturing process. Here we show that a high monocyte count in CBC is not predictive of an efficacious outcome for DC generation. Nevertheless, we find that the presence of immature granulocytes in CBC may predict decreased immunostimulation elicited by DCs and, subsequently, unsuccessful preparation of DC-based IMP. However, closer evaluation of monocyte function prior to their collection for DC generation may be considered. A surrogate marker for the immunostimulatory capacity of monocytes may be evaluated in (i) their phenotype, e.g., the level of HLA-DR or ILT-3 expression on monocytes or the proportion of particular monocyte subsets according to CD14 and CD16 expression, or (ii) their ability to produce pro-inflammatory cytokines upon TLR stimulation (27).

In summary, monocytes represent a key starting material for anti-cancer DC-based vaccine manufacture. Therefore, monocyte conditions have an impact on the manufacturing yield, the differentiation into DCs, and the level of maturation and subsequent immunostimulatory functions. For DC manufacture from heavily pretreated pediatric patients with high-risk sarcomas and neuroblastoma, we conclude that the manufacturing yield and immunostimulatory quality of anti-cancer DC-based vaccine generated from patient's monocytes were not influenced by the monocyte isolation modality but were detrimentally affected by certain combinations of anti-cancer agents. Thus, the combination of chemotherapy or targeted therapy with DC-based immunotherapy needs to be scheduled not only with respect to the likely beneficial role of anti-cancer agents on the immunogenicity of tumor antigens for both *in vitro* DC generation via induction of immunogenic cell death and *in vivo* for effector response of DC-activated T-cells but also with respect to optimal monocyte immunostimulatory functions. Finally, these findings may also have implications for the general pharmacology of anticancer treatment. As our model of *ex vivo*-activated DC preparation generally parallels the *in vivo* differentiation pathways of monocytes to the antigen-presenting cells, we may imply that drug combinations at doses used clinically may result in an impairment of patient DCs and possibly immune competence in general. In conclusion, these findings may stimulate further research on dose and mechanism-of-action-based drug combination in patient-centered trials to optimize the treatment modalities currently available in clinical oncology.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee, University Hospital Brno. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

EH contributed to the trial design, contributed to the study design, participated in clinical data acquisition and analysis, contributed to Supplementary Material preparation, and drafted the manuscript. KP supervised IMP manufacture, contributed to laboratory data acquisition and analysis, contributed to data interpretation, and drafted the manuscript. DC participated in clinical data acquisition, contributed to Supplementary Material preparation, and revised the manuscript. IS performed statistical analysis, contributed to figure preparation and data interpretation, and drafted the manuscript. PMu contributed to the trial design, performed patient enrollment and treatment, contributed to data interpretation, and drafted the manuscript. PMa contributed to the trial design, participated in patient treatment, and drafted the manuscript. LFe contributed to laboratory data acquisition and analysis, contributed to Supplementary Material preparation, and drafted the manuscript. JM contributed to the trial design and drafted the manuscript. LJ participated in IMP manufacturing and revised the manuscript. LSe contributed to IMP manufacturing—monocyte harvest and drafted the manuscript. RP contributed to IMP manufacturing—starting material harvest and revised the manuscript. LFl contributed to IMP manufacturing—certification and revised the manuscript. LSo contributed to the trial design and revised the manuscript. RD, JS, and DV contributed to the trial design, contributed to data interpretation, and revised the manuscript. LZ-D conceived the study design, designed and supervised laboratory data acquisition and analysis, contributed to data analysis and interpretation, and drafted and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01034/full#supplementary-material

Supplementary Figure 1 | Decision tree for DC-IMP manufacturing workflow including in-process and quality controls.

Supplementary Figure 2 | Number of anti-cancer therapy lines preceding monocyte harvest were compared based on QC parameters: **(A)** DC yield, and post-thaw: **(B)** viability, **(C)** DC phenotype on day 0: CD14, CD197, CD80, CD86, CD83 and on day 2: MHC II, CD80, CD86, CD83 and immunostimulatory properties presented by **(D)** IL-12 production, IL-10 production and IL-12/IL-10 production ratio, **(E)** allo-MLR and auto-MLR.

Supplementary Figure 3 | Manufacturing subgroup from monocytes harvested after MTD-based therapy potentially interfering with monocyte biology and manufacturing subgroup from monocytes from untreated patients or after non-interfering treatment compared based on QC parameters: **(A)** DC yield, and post-thaw: **(B)** viability, **(C)** DC phenotype on day 0: CD14, CD197, CD80, CD86, CD83 and on day 2: MHC II, CD80, CD86, CD83 and immunostimulatory properties presented by **(D)** IL-12 production, IL-10 production and IL-12/IL-10 production ratio, **(E)** allo-MLR and auto-MLR.

Supplementary Table 1 | Monocyte biology-interfering medications.

Supplementary Table 2 | Study patient characteristics, disease course, and therapy.

Supplementary Table 3 | Source data: CBC parameters, manufacturing details, and QC parameters.

Supplementary Material 1 | html. Interactive—medications 60 days prior to monocyte harvest.

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Assessment of Immune Response Following Dendritic Cell-Based Immunotherapy in Pediatric Patients With Relapsing Sarcoma

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Monocyte-derived dendritic cell (DC)-based vaccines loaded with tumor self-antigens represent a novel approach in anticancer therapy. We evaluated DC-based anticancer immunotherapy (ITx) in an academic Phase I/II clinical trial for children, adolescent, and young adults with progressive, recurrent, or primarily metastatic high-risk tumors. The primary endpoint was safety of intradermal administration of manufactured DCs. Here, we focused on relapsing high-risk sarcoma subgroup representing a major diagnosis in DC clinical trial. As a part of peripheral blood immunomonitoring, we evaluated quantitative association between basic cell-based immune parameters. Furthermore, we describe the pattern of these parameters and their time-dependent variations during the DC vaccination in the peripheral blood immunograms. The peripheral blood immunograms revealed distinct patterns in particular patients in the study group. As a functional testing, we evaluated immune response of patient T-cells to the tumor antigens presented by DCs in the autoMLR proliferation assay. This analysis was performed with T-cells obtained prior to DC ITx initiation and with T-cells collected after the fifth dose of DCs, demonstrating that the anticancer DC-based vaccine stimulates a preexisting immune response against self-tumor antigens. Finally, we present clinical and immunological findings in a Ewing's sarcoma patient with an interesting clinical course. Prior to DC therapy, we observed prevailing CD8+ T-cell stimulation and low immunosuppressive monocytic myeloid-derived suppressor cells (M-MDSC) and regulatory T-cells (Tregs). This patient was subsequently treated with 19 doses of DCs and experienced substantial regression of metastatic lesions after second disease relapse and was further rechallenged with DCs. In this patient, functional *ex vivo* testing of autologous T-cell activation by manufactured

DC medicinal product during the course of DC ITx revealed that personalized anticancer DC-based vaccine stimulates a preexisting immune response against self-tumor antigens and that the T-cell reactivity persisted for the period without DC treatment and was further boosted by DC rechallenge.

Trial Registration Number: EudraCT 2014-003388-39.

Keywords: dendritic cells, anticancer immunotherapy, dendritic-cell (DC)-based vaccine, pediatric sarcoma, academic clinical trials, immunomonitoring, personalized medicine

INTRODUCTION

Patients with relapsed or refractory Ewing's sarcoma have a very poor prognosis. No substantial improvement has been achieved in the therapy of sarcoma patients in the last two decades despite research, and long-term survival is still <25%. Immunotherapeutic approaches including antigen-presenting cell-based vaccines have been employed as single agent or as part of combination strategies having been substantiated by a report on immunogenicity of Ewing's sarcoma with specific translocation resulting in EWS/FLI1 fusion. Following dendritic cell (DC) vaccine with untreated autologous lymphocytes, 39% of patients had measurable immune response against a neopeptide derived from the fusion gene (1). Promising results were reported after CD25+ regulatory T-cell depletion of an autologous lymphocyte infusion product augmented with interleukin (IL)-7, where immune reconstitution correlated with an improved survival of 63% in Ewing's sarcoma and rhabdomyosarcoma (2). Immunocompetent CD8+ T lymphocytes were observed within the tumor microenvironment of metastases after DC immunotherapy (ITx) but without direct cytotoxic efficacy probably due to expression of PD-1 on lymphocytes and PD-L1 on tumor cells (3). Such immune suppression could be bypassed using recently developed anti-PD-1 and anti-PD-L1 agents, demonstrating improved survival in several malignancies, including anecdotal cases of sarcomas (4, 5).

Proper antigen presentation has a key role in directing the immune system to attack tumor cells by targeting tumor-associated antigens. We manufacture fully personalized monocyte-derived DC-based vaccines that are evaluated in an academic investigator-initiated clinical trial for children, adolescents, and young adults with progressive, recurrent, or primarily metastatic high-risk tumors (EudraCT 2014-003388-39). As a part of clinical and research evaluation of patients, we performed DC characterization, peripheral blood immunomonitoring during DC treatment, and *ex vivo* assessment of T-cell cytotoxic function pre- and post-DC treatment. During peripheral blood immunomonitoring, we quantified circulating immune cells to evaluate both positive and negative players in cancer surveillance and eradication. We focused on absolute lymphocyte count (ALC) and neutrophil-to-lymphocyte ratio (NLR). Both parameters are associated with the number of lymphocytes as key players in the immune response to tumors. Additionally, NLR reflects the number of neutrophils that is a negative prognostic factor often related to paraneoplastic immune response. The peripheral blood lymphocyte compartment contains conventional αβ TCR+ T-cells, B-cells, natural killer (NK) cells, and also minor specific effector and regulatory cell types, including regulatory T-cells (Tregs), CD56+ CD3+ NKT-like cells (6), γδ T-cells (7), and monocytic myeloid-derived suppressor cells (M-MDSCs). These immune cell subsets constitute the actual clinical immunomonitoring, and their characteristics are reviewed in **Supplementary Material 1**.

This study focuses on high-risk sarcoma patients representing a major diagnosis in this clinical trial. First, we evaluated quantitative association between basic cell-based immune parameters. Next, we described patterns of these parameters and their time changes during the DC vaccination course in the peripheral blood immunograms. As a functional testing, we evaluated immune response of patient T-cells to the tumor antigens presented by DCs in autoMLR proliferation assay. This analysis was performed with T-cells obtained prior to DC ITx initiation and with T-cells collected after administration of the fifth dose of DCs. Finally, we presented clinical and immunological findings from DC-based ITx after relapse in the case of the Ewing's sarcoma patient.

METHODS

Clinical Trial Design and Methodology

This nonrandomized, open-label, academic, investigator-initiated, phase I/II clinical trial (EudraCT No. 2014-003388-39) was performed at a single center in Czechia in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. The protocol was approved by the local ethics committee at the site and by the designated authority of Czechia (the State Institute for Drug Control).

Patients eligible for the clinical trial were children, adolescents, and young adults (1–25 years old) with histologically confirmed refractory, relapsing, or primarily metastatic high-risk tumors; Karnofsky or Lansky score ≥50; life expectancy longer than 10 weeks; and adequate function of bone marrow, kidney, liver, and heart defined as absolute neutrophil count (ANC) ≥0.75 × 10^3/μl, thrombocytes ≥75 × 10^3/μl, hemoglobin 80 g/l, estimated glomerular filtration rate (eGFR) ≥70 ml/min/1.73 m^2, serum creatinine ≤1.5-fold upper limit for the appropriate age, bilirubin ≤1.5-fold upper limit for the appropriate age,

AST and ALT ≤2.5-fold upper limit for the appropriate age, ejection fraction ≥50%, and fractional shortening ≥27% assessed by echocardiography. In the case of bone marrow infiltration, ANC had to be $\geq 0.5 \times 10^3/\mu l$ and thrombocytes $\geq 40 \times 10^3/\mu l$. In the case of liver metastases, AST and ALT must have been ≤5-fold upper limit for the appropriate age. Patients must not have had severe ongoing toxicity resulting from any previous treatment. Radiotherapy (RTx), myelosuppressive, and immunosuppressive treatment must have been withdrawn at least 3 weeks before tumor tissue harvesting; the only exception is corticoid treatment of brain edema that was allowed. Myelopoietic growth factors must have been withdrawn at least 7 days before tumor tissue harvesting. Targeted therapy must have been withdrawn at least 7 days for tyrosine kinase inhibitors (TKI) or at least 3-fold half-life of the drug (upper limit 6 weeks) before tumor tissue harvesting. The time interval between autologous transplantation and tumor tissue harvest must have been ≥12 weeks and in the case of allogeneic transplantation ≥26 weeks. Patients with seropositivity to HIV1, HIV2, *Treponema pallidum*, hepatitis B or C, known hypersensitivity to the study medication, an autoimmune disease that was not adequately treated, uncontrolled psychiatric disease, or uncontrolled hypertension were not eligible. Allowed medication prior to monocyte harvest (leukapheresis) was as follows: metronomic chemotherapy (CTx), immune checkpoint inhibitors, and anti-CD20 antibodies are allowed as concomitant medication for any time before leukapheresis. Monoclonal antibodies (except anti-CD20), high-dose CTx, and high-dose corticoids must have been withdrawn at least 3 weeks prior to leukapheresis with the exception of corticoid treatment of brain edema, which was allowed. Since November 2017, amendment of the procedure for monocyte harvest was made, and TKI must have been withdrawn according to their half-life: drugs with short half-life of 3–14 h at least 2 days before leukapheresis (axitinib, dabrafenib, dasatinib, ibrutinib, idelalisib, nintedanib, ruxolitinib, trametinib), drugs with medium half-life of 15–35 h at least 7 days before leukapheresis [alectinib, bosutinib, lapatinib, lenvatinib, nilotinib, osimertinib, pazopanib, ponatinib, regorafenib, and non-tyrosine kinase inhibitor (non-TKI) everolimus], and drugs with long half-life of 36–60 h at least 12 days before leukapheresis (afatinib, ceritinib, erlotinib, gefitinib, imatinib, cabozantinib, crizotinib, sorafenib, sunitinib, vemurafenib, and non-TKI temsirolimus). Myelopoietic growth factors must have been withdrawn at least 7 days before leukapheresis/monocyte harvest. Patients previously treated with DCs were not allowed to enter the trial.

The primary endpoint of the trial was assessment of safety by analysis of incidence of adverse events of special interest (AESI; i.e., allergic reactions grade ≥3, acute or subacute autoimmune organ toxicity symptoms manifesting up to 30 days after administration of the vaccine, injection site reactions grade ≥4, infectious complications grade ≥3). The secondary safety endpoint was incidence of all adverse events assessed in relation to type, seriousness, and causality. Secondary efficacy endpoints were time to progression, overall survival, objective response to treatment at 12 and 24 months, and clinical benefit rate assessment at 6 and 12 months.

Investigational medicinal product (IMP) was administered as an add-on therapy to standard treatment. The dose of IMP contains 2×10^6 DCs in 100 μl of cryopreservation medium. DC-based IMP was administered intradermally every 3 ± 1 weeks, up to 35 doses, to a predefined site on the left or right arm near the axillary lymph node. The evening before administration and two evenings after application, topical imiquimod, toll-like receptor (TLR)-7 agonist, was applied on the injection site as an adjuvant. On the day of administration, the patient had to have adequate bone marrow function (defined in the same way as in the entry criteria described above) and was not allowed the following therapy: more than a week systemically administered corticosteroids except treatment for cerebral or spinal edema (single administration of corticoids due to premedication, treatment of allergic reaction, and substitution treatment in secondary hypocortisolism are allowed), anticoagulants in therapeutical dose (prophylactic doses of low-molecular-weight heparins were allowed), erythropoietin, pegylated granulocyte-stimulating growth factors or other growth factors except for filgrastim, RTx to sites and regional lymph nodes, except radiation for pain control, the interval between vaccine application, and administration of conventional CTx must have been more than 72 h. Complete blood count, biochemical analysis, and immunomonitoring were performed on every patient visit associated with administration of IMP.

DC Manufacturing and Quality Control

The DC-based vaccine, called MyDendrix, was manufactured under GMP in Clean rooms of the Department of Pharmacology, Faculty of Medicine, Masaryk University. Briefly, mononuclear cells were collected by leukapheresis, and then monocytes were separated by elutriation or adherence to a plastic surface. Harvested monocytes were cultivated with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and differentiated into DC. Immature DCs were subsequently exposed to autologous tumor lysate antigens. The preparation of tumor lysate from the patient's tumor obtained during curative surgery or extended biopsy preceded monocyte harvest. Maturation was induced by lipopolysaccharide and interferon-γ. Manufactured DCs were aliquoted into IMP doses, each containing 2×10^6 DCs based on reports (8, 9), cryopreserved in DMSO-containing medium, and stored at −150°C to −196°C. Quality control (QC) of DC-based IMP included viability, cell phenotype, production of IL-12 and IL-10, and stimulation of allogeneic and autologous T-cells to reflect the level of stimulatory properties of DCs. Details on DC-based IMP manufacturing were described in **Supplementary Material 2** (8, 10). DCs were stored frozen until the day of administration when a DC dose was shipped on dry ice for administration to a study patient, shortly thawed, and immediately injected intradermally to the patient.

Ex vivo Assessment of Prevaccination and Postvaccination T-Cells

Stimulatory properties of DCs were examined pre- and post-DC treatment by autologous mixed lymphocyte reaction (MLR). Pre-DC ITx lymphocytes were obtained during the manufacturing of

DCs of from the elutriation process or adherence of leukapheresis product obtained for separation of monocytes. The number of T-cells in the lymphocyte-rich fraction was quantified by flow cytometry: approximately 10^5 PBMCs were mixed with 10 μl of anti-CD45-PC7 (clone J33) and anti-CD3-FITC (clone UCHT1, both from Beckman Coulter), incubated 20 min in the dark, and analyzed on an FC500 flow cytometer (Beckman Coulter). PBMCs were aliquoted, cryopreserved in 1,000 μl of Cryostor CS5 (BioLife Solutions), frozen, stored at −150°C to −196°C, and thawed prior to auto-MLR seeding. For post-DC treatment assay, PBMCs were obtained from peripheral blood collected into K3EDTA tube (7 ml, Sarstedt) after application of at least five doses of DCs. Blood was layered onto Histopaque-1077® (Sigma-Aldrich, density 1,077 g/ml) and centrifuged (450 g, 30 min, 20°C, acceleration 3, brake 3). Fractions of mononuclear cells were collected and washed with Hank's Balanced Salt Solution (HBSS, Lonza). 10^7 PBMCs were cryopreserved in 1,000 μl Cryostor CS5 (BioLife solutions) and stored at −150°C. For pre- and post-DC treatment autoMLR, 10^7 target lymphocytes were stained with 250 μl 10 μM carboxyfluorescein succinimidyl ester (CFSE, Sigma-Aldrich) and seeded into sterile 96-well culture plate (Sarstedt, TC Plate 96-well, Suspension, F) at 10^5 cells/well in X-vivo 10 medium (Lonza) containing 5% inactivated human male AB serum (Sigma-Aldrich) at a 1:10 effector:target ratio (10^4 DC/well), positive control (PC) with phytohemagglutinin (PHA, Sigma-Aldrich) 1 mg/ml HBSS (final concentration 10 μg/ml in MLR), or negative control (NC) with complete X-vivo medium, final volume 200 μl/well. MLR experiments were seeded in triplicates and cultured for 6 days at 37°C/5% CO_2. Then 2×10^4 cells from each well were stained with CD3-PC7 (clone UCHT1, 10 μl/test, Beckman Coulter) for flow cytometric detection of CFSE fluorescence dilution on CD3+ T-cells. Discrimination for dividing cells was set up using the NC. T-cell proliferation was calculated as follows: [(average % of dividing T-cells in 10:1 MLR)−(average % of dividing T-cells in NC)] × 100/[(average % of dividing T-cells in PC)−(average % of dividing T-cells in NC)].

The medium from autoMLR was centrifuged, and pooled supernatant from triplicates was stored at −20°C until analysis. The concentration of interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-17A was measured using a flow cytometric bead assay (BD Biosciences).

Peripheral Blood Immunomonitoring

Detailed peripheral blood immunomonitoring was performed at baseline (= before DC therapy initiation) and at each DC dose administration. The samples were collected on the day of vaccination just before the application of the vaccine. Blood was collected in a 7.5-ml S-Monovette® tube with K_3EDTA anticoagulant. Lymphocytes (ALC) and neutrophils (ANC) were measured using a Sysmex XN hematology analyzer. NLR was calculated as ANC/ALC. Immunophenotype was analyzed by multiparameter multicolor flow cytometer and software (Navios, Beckman Coulter). Diagnostic antibodies were purchased from Beckman Coulter, premixed in equal amounts in five cocktails, and stored in the dark at 2–8°C not longer than 7 days: 1/ CD14-PE (RMO52), CD15-KrO (80H5), CD11b-APC (Bear1), CD33-FITC (D3HL60.251), CD45-PB (J33), HLA-DR-PC5 (Immu357); 2/ CD3-FITC (UCHT1), CD4-PB (13B8.2), CD16-PC7 (3G8), CD56-PE (NKH-1); 3/ CD3-FITC (UCHT1), CD4-PB (13B8.2), CD27-AF750, CD45-KrO (J33), CD45RO-ECD (UCHL1), HLA-DR-PC5 (Immu357); 4/ TCR PAN γ/δ-FITC (IMMU510), TCR Vγ9-PC5 (IMMU360), TCR Vδ2-PB (IMMU 389), CD314-APC (ON72); 5/ CD3-FITC (UCHT1), CD4-PC7 (SFCI12T4D11), CD25-PC5 (B1.49.9), CD127-PE (R34.34). Blood (25 μl) was incubated with 10 μl of premixed antibody cocktail for 15 min in the dark at room temperature, hemolyzed by Versalyse® (Beckman Coulter) for 15 min and measured in five flow cytometric assays to detect: (1) M-MDSCs detected as CD45+ CD14+ CD11b+ CD33+ HLA-DR−, and their absolute count was calculated using the number of white blood cells (WBC) measured by the Sysmex XN hematology analyzer; (2) NK cells detected as CD3− CD56+ CD16+, NKT-like cells detected as CD56+CD3+; (3) circulating effector CD8+ T-cells were defined as CD3+ CD8+ CD27−, activated CD8+ T-cells were defined as CD8+ HLA-DR+; (4) γδ T-cell subsets classified as δ2+γ9−, δ2+γ9+, δ2−γ 9+, δ2−γ9− and evaluated for CD314; (5) Tregs defined as CD3+ CD4+ CD25+ CD127−/low+.

^{18}F-FDG PET/CT Scan

^{18}F-FDG PET/CT examination was performed using the hybrid scanner Biograph 64 HR+ (Siemens Erlangen, Germany). CT scan was provided in low-dose CT (25 mAs eff/120 kV). The patient had standard preparation prior to examination, including

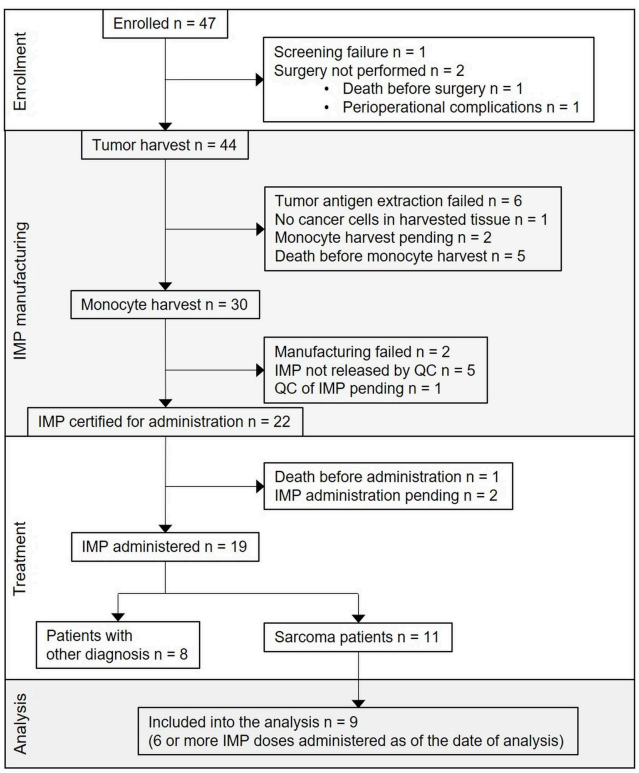

FIGURE 1 | Flow diagram for DC-based immunotherapy trial and study group definition. CONSORT flow diagram showing participant flow through each stage of the trial [enrollment, DC-based investigational medicinal product (IMP) manufacturing, treatment] and the analysis of sarcoma patients study group.

restriction of physical activity for 12 h, fasting for at least 6 h, capillary glycemia lower than 10 mmol/l (180 mg/dl) prior to ^{18}F-FDG administration and peroral hydration with 500–1,000 ml of plain water. ^{18}F-FDG was administered at a dose of 262 MBq in study 7/2017 and at a dose of 260 MBq in 1/2018. After an *in vivo* accumulation time of 60 min, whole-body scanning from the proximal third of thighs to the vertex of the skull was performed in both studies. All images were iteratively reconstructed and corrected for attenuation. ^{18}F-FDG uptake was assessed visually and also semi-quantitatively in the defined region of interest with calculation of target-to-liver ratios. A target-to-liver ratio higher than 1.0 was considered positive in all evaluated regions.

Statistical Analysis

Spearman correlation coefficient with significance test was used to measure the strength of the relationship between baseline circulating immune parameters. Graphic visualization of immunograms was performed using radar plot. Non-parametric Wilcoxon test for paired samples was used for analysis of pre- and post-treatment T-cell stimulation. *P*-values <0.05 were considered statistically significant. All statistical analyses were performed with R 3.5.3 software (11).

RESULTS

Clinical Trial Progress With Focus on Sarcoma Patients

The first subject was enrolled in September 2015. As of May 2019, the clinical trial was still ongoing, but with the accrual suspended. From the overall 47 enrolled patients, 25 (53%) were sarcoma patients. Screening failure occurred in one subject, and tumor harvest was not performed in two subjects. Tumor was harvested in 44 subjects; among them, the harvested tissue contained no cancer cells in one subject, tumor antigen extraction failure presenting as low concentration of protein in tumor lysate in six subjects, participation in the trial ended in five subjects due to disease progression and/or death, monocyte harvest has been pending in two subjects, monocyte harvest and subsequent manufacturing of DC-based IMP was performed in 30 subjects. Of the 30, manufacturing failed in two subjects, IMP did not pass quality control specifications in five subjects (four of them are sarcoma patients) (10), and 22 DC-based IMPs were released for administration to the patients. Of the 22, one subject died before IMP administration, administration has been pending in two sarcoma patients until the completion of high-dose CTx, and DC vaccine was administered to 19 subjects, including 11 sarcoma patients. Of these 11, nine patients received at least six doses of DC-based IMP as of March 2019 and were analyzed in presented immunomonitoring study (**Figure 1**). The age of sarcoma patients in the study group ranged from 10 to 24 years at the DC ITx initiation (**Table 1**). Stage of the disease in the study group at the DC ITx initiation was as follows: one (11%) in complete remission, three (33%) subjects in partial remission, one (11%) with stable disease, four (44%) with progressive disease (**Table 1**). Detail clinical course

TABLE 1 | Baseline patient characteristics and peripheral blood immune cell levels at dendritic cell (DC) therapy initiation.

Subject no./ sex	Primary diagnosis (primary localization)	Stage of the disease and PS at DC ITx init.	Age at DC ITx init.	Baseline cell-based immune parameters at DC ITx initiation									
				ALC* 10^6/ml	Eff. CD8+ %	Act. CD8+ %	NK* %	NKT-like* %	GD %* %	Tregs* %	M-MDSC count* 10^6/ml	NLR* Ratio
KDO-0101/F	Ewing sarcoma (mandible)	2nd CR Karnofsky 100	15 years	**1.9**	73.8	34.8	1.6↓	**4.9**	**2.9**	**5.9**	**0.07**	0.8↓
KDO-0102/F	Osteosarcoma (right distal femur)	PD Lansky 80	10 years	1.3↓	58.9	21.5	**4.6**	**1.9**	**4.6**	2.9↓	**0.07**	**1.2**
KDO-0114/M	Synovial sarcoma (left thigh)	PD Karnofsky 80	15 years	0.2↓	34.3	72.0	0.5↓	**2.9**	1.1↓	**12.0**	0.63↑	19.9↑
KDO-0118/F	Ewing sarcoma (spine C5-Th2)	PR Karnofsky 100	24 years	0.6↓	31.3	11.6	**8.1**	**2.2**	**3.2**	**4.5**	0.25↑	5.2↑
KDO-0119/F	Alveolar rhabdomyo-sarcoma (primum ignotum)	PR Karnofsky 80	13 years	0.6↓	25.1	10.8	**5.8**	**4.6**	**2.6**	**13.4**	**0.24**	**2.7**
KDO-0124/F	Osteosarcoma (right proximal tibia)	2nd mts relapse Karnofsky 100	19 years	0.8↓	73	21.9	**6.4**	**1.5**	**6**	2.9↓	**0.04**	**1.6**
KDO-0131/M	Embryonal rhabdomyosarcoma (pelvis)	PR Karnofsky 70	19 years	0.6↓	94.9	60.5	3.5↓	**14.8**	**3.1**	3.0↓	**0.00**	**1.7**
KDO-0133/M	Osteosarcoma (right proximal femur)	PD Karnofsky 100	24 years	0.9↓	49.1	2.7	**6.5**	**1.5**	**2.8**	3.0↓	0.26↑	**2.9**
KDO-0139/F	Osteosarcoma (left distal femur)	SD Karnofsky 90	22 years	0.5↓	14.73	37.9	4.9↓	**1.2**	0.6↓	**7.9**	0.42↑	10.7↑

*Cell-based immune parameters and their age-specific reference range (*if available): ALC, absolute lymphocyte count (reference range[1] 10 – 16 years 1.4–4.2 × 10^6/ml, >16 years 1.2–4.1 × 10^6/ml); NLR, neutrophil-to-lymphocyte ratio (reference range[2] 1–3); Ef CD8+, circulating effector cytotoxic T-cells (CD27-/CD8+, % of CD8+ T-cells); Act CD8+, activated cytotoxic T-cells (HLA-DR+/CD8+; % of CD8+ T-cells); NK cells, natural killers (reference range[1] 10–16 years 4–51% of lymphocytes, >16 years 5–49% of lymphocytes); NKT-like, circulating CD3+CD56+ cells (reference range[1] 10–16 years 0.64–15% of lymphocytes, >16 years 1–18% of lymphocytes); GD-T, gamma-delta T-cells (reference range[1] 10–16 years 2–17% of lymphocytes, >16 years 0.8–11% of lymphocytes); Treg, regulatory T-cells (reference range[1] 10–16 years 4–20% of CD4+ T-cells, >16 years 4–17% of CD4+ T-cells); M-MDSC, monocytic myeloid-derived suppressor cells (reference range[3] 0–0.24 × 10^6/ml). Numbers in bold refer to the values within the reference range, ↑–above the upper limit of the reference range, ↓–bellow the lower limit of the reference range. [1]Reference range originated from Schatorje et al. (12). [2]Estimated from reference ranges for relative differential cell blood count (13). [3]Own reference value, source group described in Pilatova et al. (13). init., initiation; F, female; M, male.*

of disease in nine sarcoma study patients is summarized in **Supplementary Material 3**.

No immune or infection-related AESIs were reported for all 15 evaluated subjects receiving DC ITx by the date of analysis.

Peripheral Blood Immunomonitoring of DC-Treated Sarcoma Patients

First, we evaluated the possible association of cell-based immune parameters in sarcoma patients before DC ITx and during DC treatment, up to six doses of DCs (**Figure 2**). Based on positive and negative correlations, immune parameters clustered *de facto* into two groups with inverse relation; a group consisting of ALC, proportion of effector cytotoxic T-cells among all T-cells, proportion of CD56+ CD3+ NKT-like cells among lymphocytes, proportion of γδ T-cells among lymphocytes, and an inversely correlated group with neutrophil-to-lymphocyte ratio (NLR), proportion of regulatory T-cells among CD4+ cells, number of M-MDSC, proportion of activated HLA-DR+ CD8+ cells among CD8+ cells, and proportion of CD56+ CD16+ CD3− NK cells among lymphocytes (**Figure 2**).

Baseline circulating immune parameters in nine sarcoma patients are shown in **Table 1**. At baseline, eight of nine patients had lymphopenia with mean ALC of 0.81 × 10^6/ml (**Table 1**). An exception was patient KDO-0101 (ALC 1.9 × 10^6/ml) with Ewing's sarcoma whose clinical course and laboratory findings are described later. The proportion of NK cells was low in six of nine patients (median 4.9%, min. 0.5%, max. 8.1%). The proportion of NKT-like cells among lymphocytes was predominantly low (median 2.2%), except for expanded NKT-like cells (14.8% of lymphocytes) in patient KDO-0131. γδ T-cells were low in six of nine patients (median 2.9%, min. 0.6%, max. 6.0%). Based on observed positive and negative association between particular cell-based immune markers, we constructed peripheral blood immunograms with putative anticancer effectors in upper part of an immunogram (namely, total lymphocytes, effector cytotoxic T-cells, CD56+ CD3+ NKT-like cells, γδ T-cells), and on the other hand, cancer-promoting or immunosuppressive actors (namely, NLR, M-MDSC, Tregs) and related factors (activated T-cells and NK cells) in the lower part of an immunogram (**Figure 3**). In peripheral blood immunograms, we presented baseline values of cell-based immune markers and their level after doses 1, 3, and 6 of ITx with DCs (**Figure 3**). The peripheral blood immunograms revealed distinct patterns in particular patients in the study group. For instance, we observed "immune-activated" pattern with patient KDO-0101 with Ewing's sarcoma who started DC ITx in the second complete remission, ALC was not decreased, effector cytotoxic T-cells represented the majority of circulating T-cells, and NLR and M-MDSC count were low. On the other hand, case KDO-0114 with progressing synovial sarcoma appeared to have an "immune-suppressive pattern" with high NLR, M-MDSC count, Tregs, and low ALC, proportion of effector cytotoxic T-cells, as well as NKT-like and γδ T-cells. Regarding time-dependent variations over the DC vaccination course, we did not observe any consistent trend in the dose-dependent change of levels of evaluated immune system parameters.

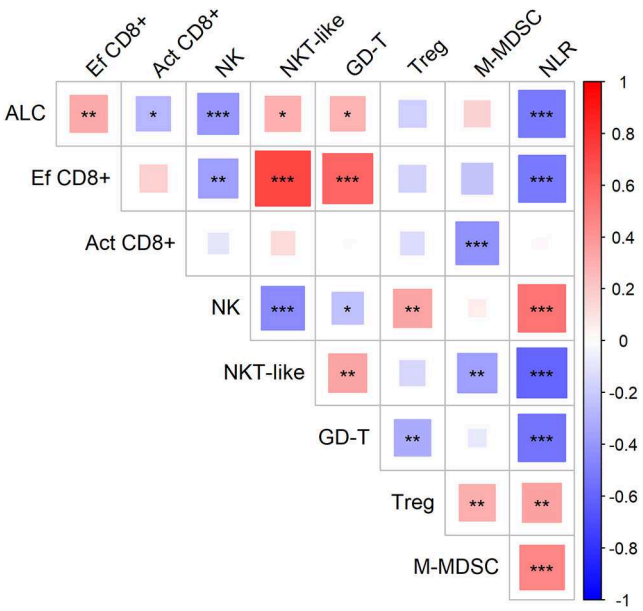

FIGURE 2 | Association of circulating immune markers during the course (from baseline to the sixth dose) of therapy with dendritic cells (DCs) in sarcoma study group. Red—positive correlation, blue—negative correlation; strength of relationship is represented by size of the square and intensity of the color, larger squares with intensified color have stronger relationship; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ALC, absolute lymphocyte count (10^6/ml); NLR, neutrophil-to-lymphocyte ratio; Ef CD8+, circulating effector cytotoxic T-cells (% of CD27− of CD8+ T-cells); Act CD8+, activated cytotoxic T-cells (% of HLA-DR+ of CD8+ T-cells); NK, circulating NK-cells (% of lymphocytes); NKT-like, circulating NKT-like cells (% of lymphocytes); GD-T, γδ T-cells (% of lymphocytes); Treg, regulatory T-cells (% of CD4+ T-cells); M-MDSC, monocytic myeloid-derived supressor cells (10^6/ml).

Patient T-Cells *in vitro* Stimulation by DCs Before and After DC Vaccination

The stimulation of sarcoma patient T-cells was examined by MLR proliferation assay with DCs from manufactured IMP and autologous T-cells obtained before DC ITx (pre-DC) and after at least five doses of DCs (post-DC) (**Figure 4**). The level of auto-MLR ranged from 0.5 to 18% (median 7.7%) with T-cells collected before DC ITx and from 4.9 to 28.4% (median 14.6%) with T-cells obtained after DC vaccination. Paired data with both pre-DC and post-DC were available for five cases, and all exhibited an increase in the T-cell stimulation after DC ITx. We observed the lowest post-DC increase in autologous T-cell stimulation by self-tumor antigens in cases KDO-0114, KDO-0124, and KDO-0133 who started DC treatment in disease progression. On the other hand, the highest increase in the T-cell stimulation with post-DC T-cells was exhibited by patient KDO-0101 who started DC ITx in complete remission of Ewing's sarcoma and remained at least up to ninth dose of DCs in complete remission. This case is described in more detail.

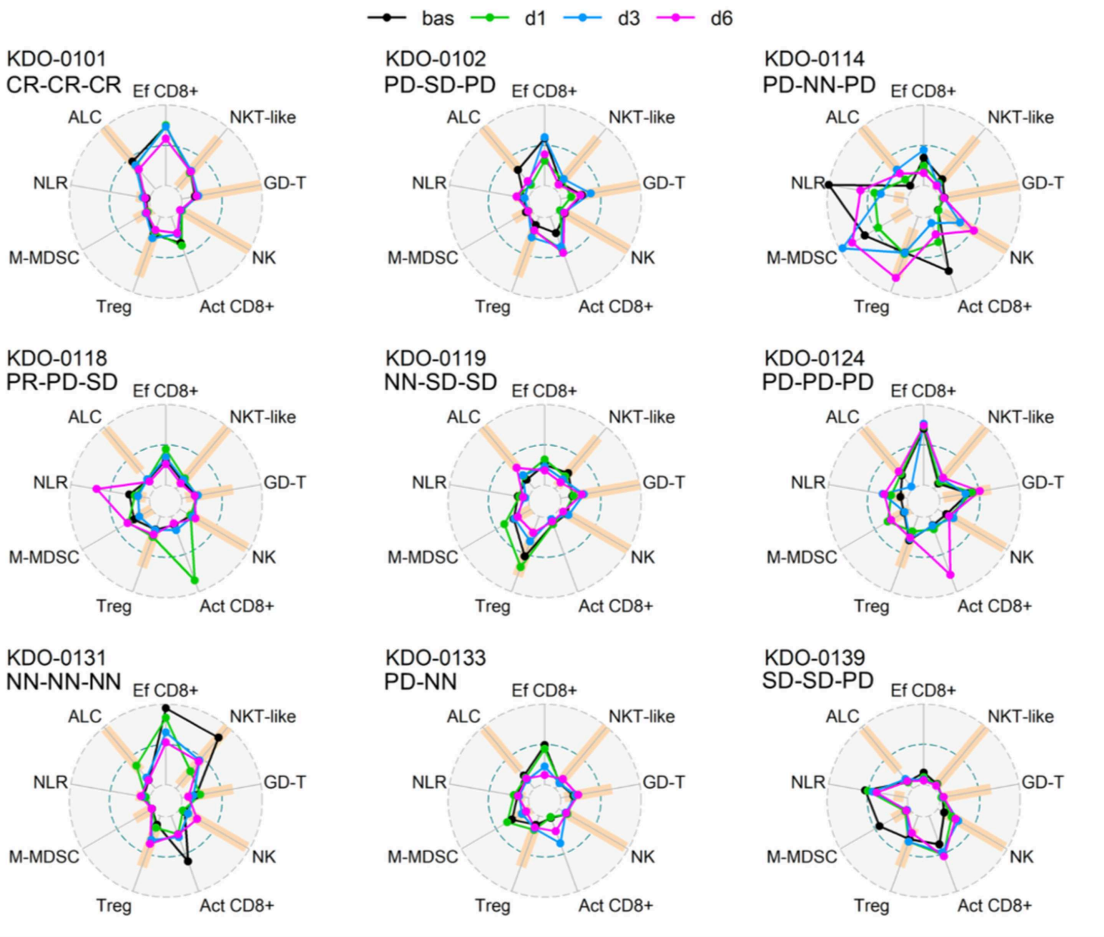

FIGURE 3 | Peripheral blood immunograms of dendritic cell (DC)-treated sarcoma patients. Nine circulating immune parameters are radially arranged with reference ranges shown in orange. Parameters are scaled according to numbers achieved within the entire study group of nine patients. Outer circle (OC, gray dashed) represents the upper limit of the reference range for ALC, NK cells, NKT-like cells, GD T-cells, maximum number reached for the particular marker for Tregs, M-MDSC, and NLR or 100% for Ef CD8+ and Act CD8+; small inner circle (IC, gray dashed) represents zero level; middle circle (MC, pacific blue dashed) represents 50% of OC level. Particular levels are listed for each parameter as follows. ALC, absolute lymphocyte count (reference range[1] 10–16 years 1.4–4.2 × 10^6/ml, >16 years 1.2–4.1 × 10^6/ml; OC: 4.2 10^6/ml); NLR, neutrophil-to-lymphocyte ratio (reference range[2] 1–3; OC 19.9); Ef CD8+, circulating effector cytotoxic T-cells (CD27−/CD8+; % of CD8+ T-cells) (OC: 100%); Act CD8+, activated cytotoxic T-cells (HLA-DR+/CD8+; % of CD8+ T-cells) (OC 100%), NK cells (reference range[1] 10–16 years 4–51% of lymphocytes, >16 years 5–49% of lymphocytes; OC: 51% of lymphocytes); NKT-like, circulating CD3+CD56+ NKT-like cells (reference range[1] 10–16 years 0.64–15% of lymphocytes, >16 years 1–18% of lymphocytes, OC 18% of lymphocytes); GD-T, γδ T-cells (reference range[1] 10–16 years 2–17% of lymphocytes, >16 years 0.8–11% of lymphocytes; OC: 17% of lymphocytes); Treg, regulatory T-cells (reference range[1] 10–16 years 4–20% of CD4+ T-cells, >16 years 4–17% of CD4+ T-cells; OC: 25.3% of CD4+ T-cells); M-MDSC, monocytic myeloid-derived suppressor cells (reference range[3] 0–0.24 × 10^6/ml; OC: 0.98 × 10^6/ml). Baseline levels prior to DC ITx initiation are shown in black and levels at doses d1, d3, d6 are shown in shades of blue. Clinical outcome is shown for each subject at DC ITx initiation, at dose 5, at dose 9. Clinical outcome is abbreviated as follows: CR, complete response; PD, progressive disease; SD, stable disease; NN, non-CR/non-PD; NA, not available. [1]Reference range originated from Schatorje et al. (12). [2]Estimated from reference ranges for relative differential cell blood count. [3]Our user-defined reference value, source group described in Pilatova et al. (13).

DC-Based Therapy After Relapse in a Ewing's Sarcoma Patient: Treatment Course and Outcome

A girl, born 2001, was diagnosed with primary disseminated EWS/FLI-1 positive Ewing sarcoma with a primary tumor in the mandible and skull metastases in December 2011. The patient was treated by protocol EuroEwing 2008, 6x VIDE: vincristine (1.5 mg/m^2/day; day 1), ifosfamide (3,000 mg/m^2/day; days 1, 2, 3), doxorubicin (20 mg/m^2/day; days 1, 2, 3), etoposide (15 mg/m^2/day; days 1, 2, 3), 1× VAC: vincristine (1.5 mg/m^2/day; day 1), actinomycin (0.75 mg/m^2/day; days 1, 2), cyclophosphamide (1,500 mg/m^2/day; day 1) from 12/2011 to 10/2012. Surgery was performed in June 2012 with partial resection of primary tumor. Radical resection was not possible due to mutilation. High-dose (HD) CTx treosulphan/melphalan with autologous peripheral blood stem cell transplantation (APBSC) followed in July 2012. Then, the patient underwent RTx of the mandible and parietal bone from September 2012 to November 2012 (34 Gy + 45 Gy), and CTx continued by

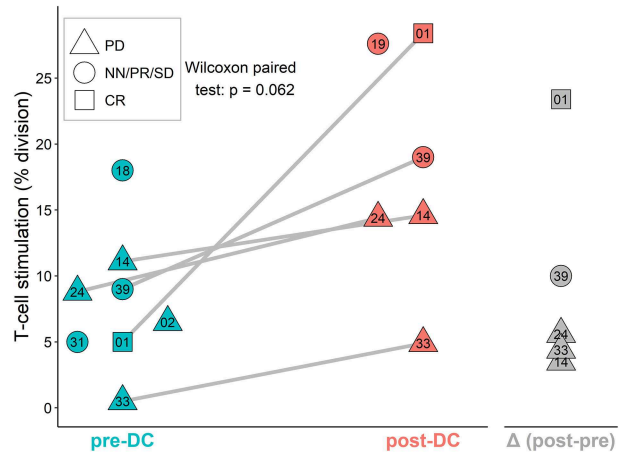

FIGURE 4 | AutoMLR with patients' pre-dendritic cell (DC) and post-DC T-cells stimulated by DC-based investigational medicinal product (IMP). The stimulation is expressed as the percentage of dividing autologous T-cells after incubation with DCs. Pre-DC (blue) refers to the stimulation of patients' T-cells obtained prior to DC-based ITx initiation. Post-DC (red) refers to the stimulation of patients' T-cells obtained after the fifth dose of DC vaccine. The difference (post-DC)−(pre-DC) is shown in gray. The shape of symbols refers to a stage of the disease; PD, progressive disease; CR, complete remission; NN, non-CR/non-PD; PR, partial remission; SD, stable disease. Two-digit numbers refer to the last digits in patients' number (e.g., 01 = KDO-0101, etc.). A pair of pre-DC and post-DC autoMLR in the same patient is linked by a gray line.

protocol EuroEwing 2008 with 7× VAC from October 2012 to May 2013. The first complete remission was achieved and lasted until May 2015 when the first relapse occurred in the skull. The patient was enrolled in the DC clinical trial, and the surgically removed tumor from the skull was used as a source of tumor antigens. In the second-line CTx, the patient received vincristine (1.5 mg/m^2/day; 5 days block), irinotecan (50 mg/m^2/day; 5 days block), and pazopanib (200 mg/daily). Monocytes were harvested in January 2016, and 35 doses of DC-based medicinal product were manufactured. One week after monocyte separation, palliative RTx on lesions in the skull was started and was performed from January 2016 to February 2016 with a total dose 41 Gy. Subsequently, after recovery from HD CTx and RTx, experimental DC-based ITx (on a biweekly basis) with immunomodulation *via* low-dose cyclophosphamide (26 mg/m^2/day) started in August 2016. The patient received 19 doses of DCs until the second relapse in 7/2017 with multiple metastases in the skull, pelvis (**Figures 5A,B**), and lesions in liver. FDG PET positivity without CT scan correlates was noted in the spinal column. Third-line CTx with topotecan (0.75 mg/m^2; 5 days block), cyclophosphamide (250 mg/m^2; 5 days block), and zoledronate (4 mg/4 weeks) with concomitant RTx was initiated. Evaluation of response showed stable disease. After three cycles, CTx was stopped due to hematological toxicity. Surprisingly, during the subsequent 4 months without treatment, substantial regression of metastases was noted both on PET/CT scan in 1/2018 (**Figures 5C,D**) and upon clinical examination of palpable metastases. Fourth-line maintenance metronomic CTx with low-dose vinblastine (3 mg/m^2/day) and continuing zoledronate (4 mg/dose/4 weeks) was started with rechallenge with DC-based vaccines from the original manufacturing from March 2018 to August 2018. Unfortunately, the partial regression was temporary, and slow continuing progressive disease led to the death of the patient in November 2018.

DC-Based Therapy After Relapse in a Ewing's Sarcoma Patient: *Ex vivo* Prevaccination and Postvaccination T-Cell Response and Peripheral Blood Immunomonitoring

Pre-DC treatment T-cell response evaluated by autoMLR as a part of DC quality control resulted in a mean of 5% T-cell division. Post-DC (after the fifth dose) autoMLR exhibited 28% T-cell division (**Figure 6A** blue). Production of cytokines (IFN-γ, TNF-α, IL-17A) during auto-MLR mildly increased in post-DC compared to pre-DC evaluation (**Figure 6B** blue). AutoMLR with T-cells collected before restart of DC treatment in February 2018 (after the third-line Ctx with topotecan, cyclophosphamide, and zoledronate with RT and an additional 4 months with no antitumor treatment) exhibited 22% T-cell division and, upon the fifth "rechallenge" dose, 40% T-cell division was observed (**Figure 6A** red). IFNγ production during autoMLR substantially increased after the fifth dose of DC rechallenge (**Figure 6B** red). The variations of circulating immune markers exhibited only minor changes at the beginning of both lines of therapy with DCs (**Figure 6C**). Levels of circulating immune markers at each dose of both lines of DC-based therapy are shown in **Supplementary Material 4**. At DC rechallenge, an increase in the proportion of circulating effector CD8+ cells and an increase in the proportion of γδ T-cells compared to the initiation of first-line DCs was observed (**Figure 6C**). In this patient, γδ T-cells were predominantly Vγ9-Vδ2- prior to DC ITx initiation (baseline 39%). Vγ9+Vδ2+ T-cells represented 33% of γδ T-cells, and their proportion decreased during DC Itx, and this γδ subset was almost depleted from circulation after third-line CTx (**Figure 6D**). In contrast to the Vγ9+Vδ2+ subset, Vγ9-Vδ2- T-cells were predominantly CD314(NKG2D)+ (**Supplementary Material 4**).

DISCUSSION

The primary endpoint of the clinical trial investigating anticancer therapy with DCs was the evaluation of treatment safety with interim result from 15 patients of no immune- or infection-related adverse events. Moreover, to gain more information from DC-treated patients, we performed immunomonitoring at baseline and at each DC dose. Collected data will be evaluated in the context of clinical outcomes after completion of the trial.

Here we show that an ALC was positively associated with the proportion of effector CD8+ cytotoxic T-cells out of total T-cells that is reflected by an inversion of the CD4:CD8 ratio and proportion of effector cells CD8+ among total CD8+ cytotoxic T-cells. The proportion of effector CD8+ cytotoxic

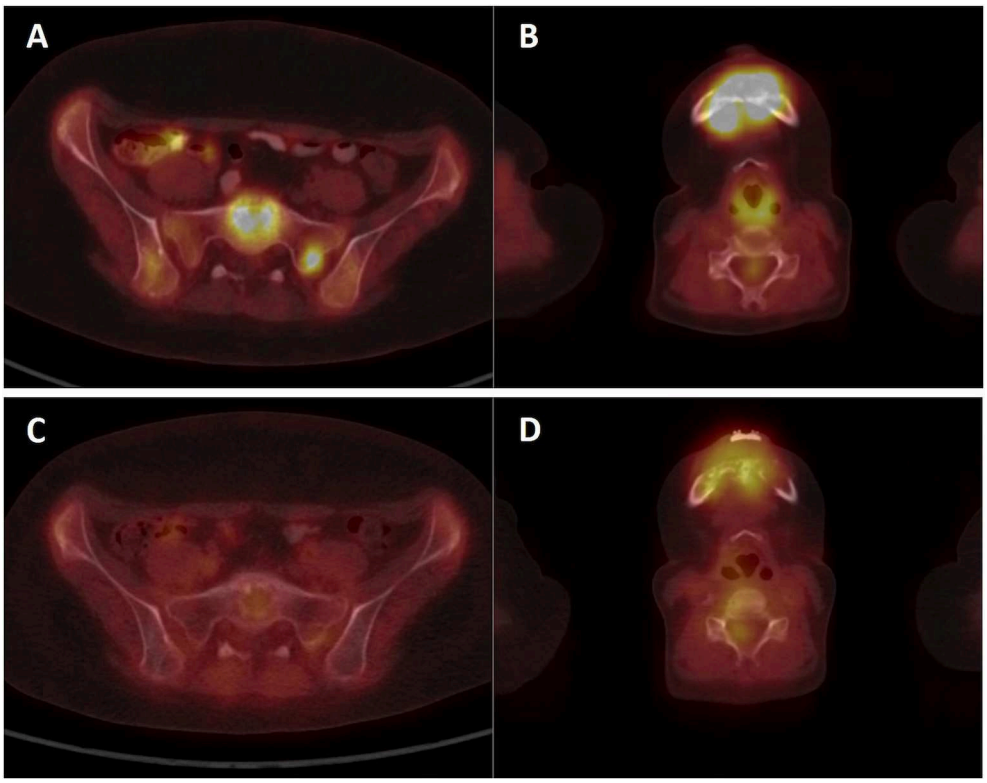

FIGURE 5 | PET/CT imaging of patient KDO-0101. **(A,B)** Examination of patient at second relapse in July 2017 showed ^{18}F-FDG-positive osteolytic lesions in the skeleton **(A)** sacrum, sacral base with a target-to-liver ratio of 2.74 and sacral left lateral mass with a target-to-liver ratio of 2.39 **(B)** mandible with a target-to-liver ratio of 4.88. **(C,D)** Control ^{18}FDG-PET/CT examination in January 2018 showed a decrease or complete diminishment of ^{18}FDG accumulation **(C)** sacrum, sacral base with a target-to-liver ratio of 0.69, and sacral lateral mass with a target-to-liver ratio of 0.66 **(D)** mandible with a target-to-liver ratio of 1.47.

T-cells among total T-cells was further correlated with the proportion of NKT-like cells and γδ T-cells. Both of these non-classical lymphocyte subsets have been studied and described for their role in cancer surveillance (6, 14, 15). On the other hand, in the putative cancer-enhancing/immune-suppressive cluster, we observed an association between circulating M-MDSC and Tregs that might be explained by increase in Tregs induced by MDSC-derived immunosuppressive cytokines (16) as described previously in non-cancer settings (17, 18). NLR associated with M-MDSC and Tregs, which may reflect "emergency" myelopoiesis induced by tumor or by host-related conditions, that promotes production of not only classical myeloid cells such as neutrophils and monocytes but also myeloid-derived suppressor cells (19). In line with two inversely associated clusters of immune-based circulating biomarkers, we have previously shown a negative correlation between effector CD27− cytotoxic CD8+ T-cells and number of both CD33hi PMN-MDSCs and M-MDSC in pediatric cancer patients (19).

The current clinical trial was designed for patients with progressive, recurrent, or primarily metastatic high-risk tumors that are always heavily pretreated by prior multimodal anticancer therapy. Indeed, patients with measurable disease represented vast majority of cases enrolled to this clinical trial. Therefore, we may expect that patients evaluated in this clinical trial exhibit prior profound suppression of immune function. Indeed, the majority of sarcoma patients were lymphopenic. On peripheral blood immunograms, we showed distinct patterns of immune parameters such as prevailing CD8+ T-cell stimulation in patient KDO-0101 or marked immunosuppression in KDO-0114. However, observations from immunomonitoring and clinical course in the patient KDO-0101 are worth particular attention. In comparison to the rest of the study group, patient KDO-0101 exhibited a lymphocyte count within the reference range, a high proportion of effector T-cells, and low levels of all observed parameters associated with adverse disease outcome, namely, Treg count, M-MDSC count, and neutrophil-to-lymphocyte ratio. This DC-vaccinated patient experienced substantial regression of metastatic Ewing's sarcoma after the second relapse. In comparison to the initial DC vaccination, at DC rechallenge, a proportion of effector and activated DC increased, although ALC dropped. We also observed an increase in γδ T-cells, which may be attributable to therapy with zoledronic acid that was part of the third-line therapy prior to DC rechallenge. Zoledronic acid causes accumulation of isopentenyl pyrophosphates (IPP), leading to stimulation of γδ T-cells (20). γδ T-cells responding to zoledronic acid are Vγ9+Vδ2+ T-cells that sense IPP via Vδ2 TCR (20).

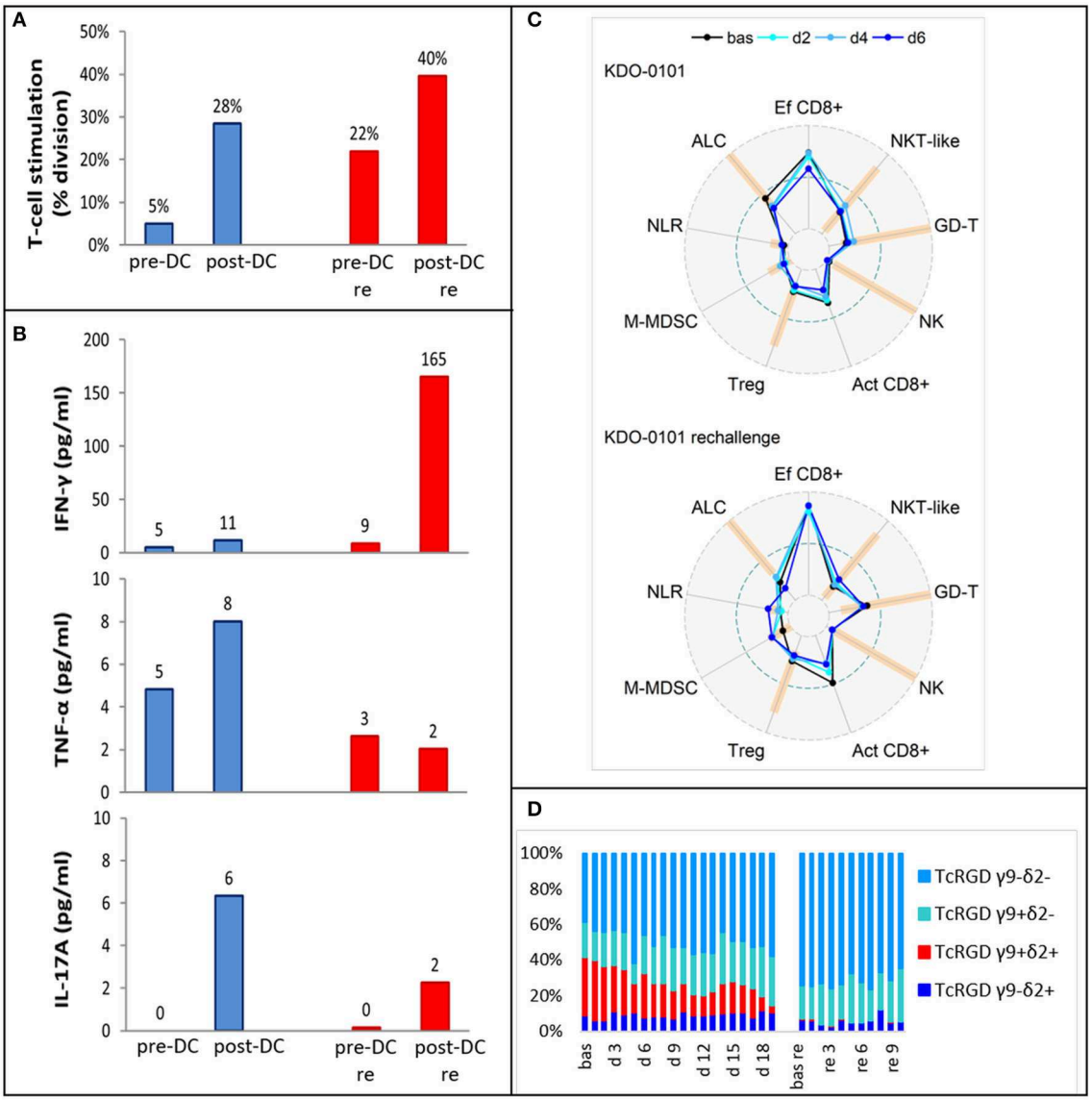

FIGURE 6 | *Ex vivo* functional and peripheral blood immunomonitoring of subject KDO-0101 during first dendritic cell (DC) immunotherapy and its rechallenge. **(A)** Stimulation of T-cells by DCs, reflected by the percentage of division T-cells. **(B)** Production of interferon-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-17A. **(A,B)** Pre- and post-DC treatment T-cell response was measured i/ (blue) before start of DC administration (pre-DC) and after the fifth dose (post-DC) ii/ (red) after 4 months with no antitumor treatment, before start of DC rechallenge (pre-DC re) and after the fifth rechallenege dose (post-DC re). **(C)** Peripheral blood immunogram from baseline (bas) through doses 2, 4, and 6 in the course of both DC treatment (upper) and DC rechallenge (lower). The layout of immunograms is described in **Figure 3**. **(D)** Four subtypes of gamma-delta TCR (Vγ9−Vδ2−, Vγ9+Vδ2−, Vγ9+Vδ2+, Vγ9−Vδ2+) in the course of both DC treatment from baseline to dose 19 and DC rechallenge from baseline to dose 10.

Interestingly, however, in this patient, we observed an increase in number of Vγ9−Vδ2− T cells and depletion of Vγ9+Vδ2+ T-cells. It is of note that only in two out of nine pediatric sarcoma patients (KDO-0118 and KDO-0139), the Vγ9+Vδ2+ subset represented a majority of circulating γδ T-cells. This is an unexpected observation in the context of reported findings (21) and of our observations in adult carcinoma patients (7) and patients treated and evaluated in the DC clinical trial with non-sarcoma cancers (data not shown).

The second relapse in subject KDO-0101 occurred during maintenance therapy with DC ITx. The observed temporary regression of metastases of the Ewing's sarcoma after second relapse may have been related to the immune response induced by previous DC treatment. Despite stable disease on the third-line CTx topotecan/cyclophosphamide, the patient exhibited partial response after concomitant RTx and DC vaccination only. Performance status of the patient was good over a long period of time, namely, Karnofsky index over 80%, despite

heavy metastatic involvement in skull, pelvic bones, spinal column, and liver. Performance status declined after 1 year of RTx, DCs ITx, and metronomic vinblastine and zoledronic acid. This unexpected observation suggests an opportunity to deliver such treatment to more patients. We observed substantial enhancement of T-cell reactivity toward DC-presented tumor antigens upon DC vaccination in patient KDO-0101 and to a lesser extent in four other sarcoma patients vaccinated with DCs and analyzed here. Thus, we confirmed that our anticancer DC-based vaccine stimulates a preexisting immune response against self-tumor antigens. Moreover, in the case of KDO-0101, functional *ex vivo* testing revealed that T-cell reactivity toward DC-presented self-tumor antigens persisted for a long period of time without DC treatment and was further boosted by DC rechallenge. In principle, the mechanism of action of anticancer DCs relies on stimulation of T-cell-mediated antitumor immune response targeting the presented cancer neoantigens. However, to date, the majority of patients treated with investigational DCs including the pediatric cancer patients in this clinical trial were end-stage or advanced cancer patients with extensive tumor mass and severely destroyed immune system. Limited clinical response achieved by DC-based ITx across numerous clinical trials can be attributed to both tumor-induced immunosuppression and, in heavily pretreated patients, also to anticancer therapy-induced immunosuppression. This is, nevertheless, supported by limited observational experience that enhancement of T-cell response to self-tumor antigens was related to the stage of the disease, that is, lower in cases with sarcomas in progression. It is thus crucial to overcome the immunosuppressive barrier to improve the efficacy of DC-based ITx as to have the antigen-presenting DC-based ITx combinable with cytokines, immune adjuvants, CTx, targeted therapy, and/or checkpoint inhibitors in order to boost T-cell effector functions and/or inhibit immune-suppressive pathways in the tumor mass (22). Ideally, selection of the right concomitant treatment to be combined with DC ITx shall be personalizable to target either particular immunosuppressive elements prevailing or particular immune effectors deficient in a particular patient, such as low-dose cyclophosphamide to deplete Tregs (23) or zoledronic acid to enhance γδ T-cells (24). In this context, immune-based biomarkers within the tumor microenvironment (if accessible) and/or systemic from peripheral blood could be exploited not only to provide an optimal ITx combination but also to select patients that would benefit from DC-based ITx. Regarding tumor-induced immunosuppression that is dependent on the tumor volume renders DC ITx less effective in patients with extensive tumor burden (25) and elicits higher tumor-specific immunologic response rates in the adjuvant compared to the metastatic setting (26). Thus, there is a rationale for the use of DC-based ITx earlier in the course of disease when tumor burden is still minimal; for example, in the adjuvant setting in patients at high risk of recurrence or in patients with minimal metastatic disease.

From our perspective beyond the study, anticancer DC vaccination could be more effective if appropriately personalized not only in terms of loading DC with self-tumor antigens but also in terms of (i) selection of the right patients that would benefit from ITx (such as patients with tumor with high mutational load), (ii) treatment at the right time when the disease and the level of immune suppression is minimal, and (iii) selection of right (possibly personalized) concomitant treatment that allows the optimal immunostimulation and anticancer activity of effector cells.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee, University Hospital Brno. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin or by the adult participants. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LFe contributed to the study design, performed laboratory data acquisition and analysis, prepared figures, tables, supplementary material, contributed to data interpretation, and drafted the manuscript. PMu contributed to the trial design, performed patient enrollment and treatment, contributed to data interpretation, and drafted the manuscript. KP supervised IMP manufacturing, contributed to laboratory data acquisition and analyses, supplementary material preparation, and drafted the manuscript. IS performed statistical analysis, contributed to figure preparation, data interpretation, and drafted the manuscript. JM contributed to the trial design, participated in clinical and manufacturing data analysis, and drafted the manuscript. ZR performed PET/CT data acquisition, contributed to figure preparation, data interpretation, and drafted the manuscript. DV and EH contributed to the trial design, data interpretation, and revised the manuscript. DC participated in clinical data acquisition, contributed to supplementary material preparation, and revised the manuscript. LFa participated in clinical data acquisition and revised the manuscript. PMa and ZP contributed to the trial design, participated in patient treatment, and revised the manuscript. RD and JS contributed to the trial design, contributed to data interpretation, and revised the manuscript. LZ-D conceived the study design, designed and supervised laboratory data acquisition and analysis, contributed to data analysis and interpretation, and drafted and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01169/full#supplementary-material

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

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Comprehensive Molecular Profiling for Relapsed/Refractory Pediatric Burkitt Lymphomas—Retrospective Analysis of Three Real-Life Clinical Cases—Addressing Issues on Randomization and Customization at the Bedside

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In order to identify reasons for treatment failures when using targeted therapies, we have analyzed the comprehensive molecular profiles of three relapsed, poor-prognosis Burkitt lymphoma cases. All three cases had resembling clinical presentation and histology and all three patients relapsed, but their outcomes differed significantly. The samples of their tumor tissue were analyzed using whole-exome sequencing, gene expression profiling, phosphoproteomic assays, and single-cell phosphoflow cytometry. These results explain different treatment responses of the three histologically identical but molecularly different tumors. Our findings support a personalized approach for patient with high risk, refractory, and rare diseases and may contribute to personalized and customized treatment efforts for patients with limited treatment options like relapsed/refractory Burkitt lymphoma.

SUMMARY

The main aim of this study is to analyze three relapsed Burkitt lymphoma patients using a comprehensive molecular profiling, in order to explain their different outcomes

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and to propose a biomarker-based targeted treatment. In cases 1 and 3, the tumor tissue and the host were analyzed prospectively and appropriate target for the treatment was successfully implemented; however, in case 2, analyses become available only retrospectively and his empirically based rescue treatment did not hit the right target of his disease.

Keywords: Burkitt lymphoma, targeted therapy, precision medicine, theranostics, pediatric oncology

INTRODUCTION

Burkitt lymphoma is a highly aggressive mature B-cell lymphoma commonly associated with translocation of *MYC* gene. The disease is classified as sporadic, endemic, or immunodeficiency related. In pediatric oncology, current standard intensive chemotherapy with anti-CD20 antibody regimens achieve long-term, disease-free survival in almost 95% of patients (1). However, a subset of patients who do not respond to the first-line chemotherapy and who experience relapse have very poor prognosis despite high-dose chemotherapy followed by stem cell transplantation (2). This subset of patients, for whom further chemotherapy-based therapies are futile, is recently often considered for therapies based on molecular analysis of their tumor tissue. We present three cases of relapsed Burkitt lymphoma. Cases 1 and 3 were treated with a therapy that reflected the molecular signature of the child's tumor, but in case 2, the therapy "missed" the target because his molecular signature was not known at the time retrieval therapy was initiated. The findings suggest that molecular signatures are unique, and a tissue biomarker-based customized therapy may be the better approach to address these poor prognosis patients than just another biomarker agnostic randomized trial.

METHODS

A comprehensive molecular profiling consisted of whole-exome, gene expression profiling and a profile of phosphorylated proteins and single-cell phosphoflow cytometry of three cases of relapsed pediatric Burkitt lymphoma searching for biological rationale for different responses to the therapy and different clinical outcomes.

Whole-Exome Sequencing

Whole-exome sequencing (WES) using the TruSeq DNA Exome Kit, the NextSeq 500/550 Mid Output Kit v2.5, and a NextSeq 500 sequencing device (all Illumina, CA, USA) was done in all three cases. Input material was 400 ng of DNA obtained from the peripheral blood (for germline exome) and formalin-fixed, paraffin-embedded (FFPE) tumor sample with $\geq$20% cancer cell count measured in the surface area of tissue slides for somatic exome. WES was done with high coverage where at least 90% of targeted regions were covered 20 times.

Gene Expression Profiling (Transcriptome Examination)

Gene expression profiling using the Affymetrix GeneChip Human Gene 1.0. ST Array (Applied Biosystems, CA, USA) was done in all three cases. Input material was 250 ng of RNA obtained from frozen tumor tissue. Samples were prepared using the GeneChip WT PLUS Reagent Kit (Affymetrix, CA, USA) according to the manufacturer's protocol. Subsequently, chips were hybridized using the GeneChip Hybridization Oven, washed using the GeneChip Fluidics Station, and scanned on the GeneChip Scanner (all Affymetrix, CA, USA), and CEL files were generated. Data were processed using R software version 3.3.3 (3). Gene expressions of 220 selected genes were subsequently compared to accumulated normal tissue samples as described previously (4), utilizing two comparator sets: one consisting of 408 normal tissue samples of different diagnoses (main general comparator) and one consisting of 5 samples of normal germinal center B cells (complementary-specific comparator). Samples were downloaded from Gene Expression Omnibus and ArrayExpress databases, and names of the database samples are listed in **Supplementary Material 1**. Expression data were calculated as Robust Multichip Average (RMA) with background correction and quantile normalization implemented in rma function in oligo package (5). Difference of expression of each gene was calculated as fold change (FC) from the mean of the comparator set and tested using a two-sided one-sample *t*-test, with false discovery rate (FDR) adjustment applied. An FC value of 0.5 and more was considered important. No specific *p*-value was considered limiting the discrimination of differently expressed genes with FC $>$ 0.5. Utilizing the general comparator consisting of 408 samples offers highly significant results corresponding to the power of 10 to -25 for the FDR-adjusted *p*-values for most of the evaluated genes with FC of 0.5 or more, and rising to the power of 10 to -100 for the FDR-adjusted *p*-values for genes with FC $>$ 2.

RNA transcription data from the tumor tissues were analyzed as well using Biogrid (http://thebiogrid.org), and http://www.genome.jp/kegg/pathway.html and mathematical simulations of protein–protein interactions as described before (6).

Profile of Phosphorylated Proteins

Human Phospho-RTK Array Kit (R&D Systems) was used to determine the relative levels of tyrosine phosphorylation of 49 different RTKs. Human Phospho-MAPK Array Kit (R&D Systems) was employed for the detection of phosphorylation status of 26 MAPKs, serine/threonine kinases, and other signaling proteins. Both arrays were performed as previously described (7).

Single-Cell Phosphoflow Cytometry

Peripheral blood mononuclear cells (PBMCs) were separated on Ficoll-Paque (GE Healthcare) according to the manufacturer's

instructions. PBMCs were reconstituted in a culture medium consisting of RPMI 1640 with 25 mM HEPES, L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Lonza, Basel, Switzerland) to a final concentration of 2 million cells per milliliter. After a 1-h rest at 37°C in a 5% CO_2 atmosphere, the cells were stimulated on 96-well plate containing coated anti-CD3 (10 μg/ml, Exbio Praha) and free costimulatory anti-CD28/CD49d antibodies (1 μg/ml, BD Biosciences) for 5, 15, and 30 min. The cells were fixed with 4% formaldehyde for 10 min and permeabilized with ice-cold methanol for 30 min. The following fluorochrome conjugates were used for cytometric detection: phospho-Akt (Ser473)-Alexa Fluor 488, phospho-S6 (Ser235/236)-Pacific Blue (Cell Signaling Technologies), phospho-mTOR (Ser2448)-PE (eBioscience, Thermo Fisher), CD45-Pacific Orange, CD45RA-APC (Exbio), CD8-PE-Cy7 (Beckman Coulter), CD4-PerCP-Cy5.5, and CD3-APC-H7 (BD Biosciences). The samples were acquired on Canto II flow cytometer and analyzed using FlowJo software (BD Biosciences).

RESULTS

Case 1

A 7-year-old previously healthy boy presented with $t_{(8;14)}$ positive abdominal stage III Burkitt lymphoma (St. Jude staging system). The boy was initially treated as per the standard BFM B-NHL Registry 2012 protocol with the addition of rituximab according to the most recent published literature (1). He responded well to the therapy and achieved a very good partial response after two cycles. His clinical course was complicated by an episode of duodenal obstruction/intussusception requiring surgical intervention. The histology from this resection revealed sclerosing mesenteritis with no evidence of lymphoma, congruent with the conclusion of a study using ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (FDG PET/CT) that revealed a very small residual tumor with only borderline FDG PET avidity. Unfortunately, the patient had disease progression 6 weeks following the completion of protocol therapy (and 3 months from the second surgery) with a new lesion within the tumor resection margin and a new mediastinal mass. A biopsy of the abdominal lesion confirmed the recurrence of Burkitt lymphoma with persistent areas of sclerosing mesenteritis.

As sclerosing mesenteritis has been associated in the literature not only with B-cell lymphomas but also with activation of the PI3K-delta pathway and immunodeficiency (8, 9), a candidate testing for this specific mutation was performed.

In the tumor, there was proven disruption of MYCC and IgH in 97% of cells according to fluorescence *in situ* hybridization (FISH). Karyotype of the tumor showed 46 chromosomes with complex changes. A germline variant of c.935C>G (p.S312C) in the PI3K-delta subunit was found both in the child and in the father. The patient's older sister and mother were negative for this variant. We tested the intracellular signaling downstream of PI3K using flow cytometry assessment of phosphorylation of Akt, mTOR, and S6 proteins in the patient's peripheral blood T-lymphocytes and detected increased basal and T-cell receptor (TCR)-induced activation (**Figure 1A**). Similarly, increased levels of PI3K were confirmed by RNA transcriptome analysis of the tumor tissue with Affymetrix GeneChipST 1.0. This analysis also revealed an increased expression of HR23B, a predictor of response to histone deacetylase (HDAC) inhibitors. Immunohistochemistry revealed a strong expression of PD-1L. The variant p.S312C has been described previously as mutation in brain cancer cell line and prostate cancer cell line (10) but has been classified as benign for development of immunodeficiency according to the ClinVar database. The allele frequency ranges between 0.008 and 0.030 in population databases (gnomAD 0.02, ExAc 0.0217, 1000G/ALL 0.008, 1000G/EUR 0.029) and was found to be 0.018 in our cohort of 508 cord blood samples (not published). Thus, this variant cannot be considered pathogenic. However, it may predispose the PI3K pathway to be activated, if other genetic and/or non-genetic factors are present.

Interestingly, even though the biopsy at the time of initial diagnosis had been tested for *TP53* and no alteration of the gene was found, in the biopsy obtained from the relapse, a new *TP53* R273C somatic mutation was identified in the tumor.

Retrieval therapy was administered with obinutuzumab 550 mg/m^2, ibrutinib 140 mg/m^2, and two cycles of ifosfamide, carboplatin, and etoposide (ICE) chemotherapy. The patient had further progression on this therapy, and a more molecular biomarker-driven theranostic approach was discussed. The therapy was changed to a single-agent window using a specific inhibitor of PI3K idelalisib 200 mg/m^2/d. In 2 weeks, we were able to document a markedly decreased PI3K pathway activation in the patient's peripheral blood T-lymphocytes (**Figure 1B**), but the disease was still showing further radiological progression. Therapy with idelalisib was not discontinued, and ibrutinib 140 mg/m^2 daily was reintroduced. Based on the transcriptome analysis, valproic acid for HDAC inhibition aiming for serum levels of 80–100 μg/ml was added, and nivolumab at 3 mg/kg every second week and metronomic cyclophosphamide at 25 mg/m^2/7 days on/7 days off were introduced for immune modulation. To support local disease management and support the tumor antigen presentation, the patient received 21-Gy radiation to the site of the abdominal relapse. There was evidence of partial remission on FDG PET/CT 3 months later and stable disease 6 months later. Due to persistence of a viable tumor on FDG PET/CT and high toxicity of allogenic stem cell transplant reported in nivolumab-treated patients (11), this approach was not considered as treatment of choice. Consequently, personalized immunotherapy with dendritic cell-based vaccine was preferred to support the antitumor immunity, and treatment with dendritic cells loaded with whole tumor lysate according to phase I/II protocol (EudraCT No. 2014-003388-39) (12) was initiated. The residual tumor resected after 11 months of such therapy consisted of mainly necrotic tissue with lymphocytic infiltration with no evidence of viable tumor. Considering that the child had achieved complete remission, valproic acid, ibrutinib, and idelalisib were gradually discontinued and the patient is continuing to take biweekly intradermal applications of autologous dendritic cell vaccine and nivolumab until May 2018 when all his 37 manufactured doses of dendritic cell-based vaccine were used up.

The progression-free survival (PFS) of 46 months following a customized, tumor tissue molecular analysis-guided regimen was

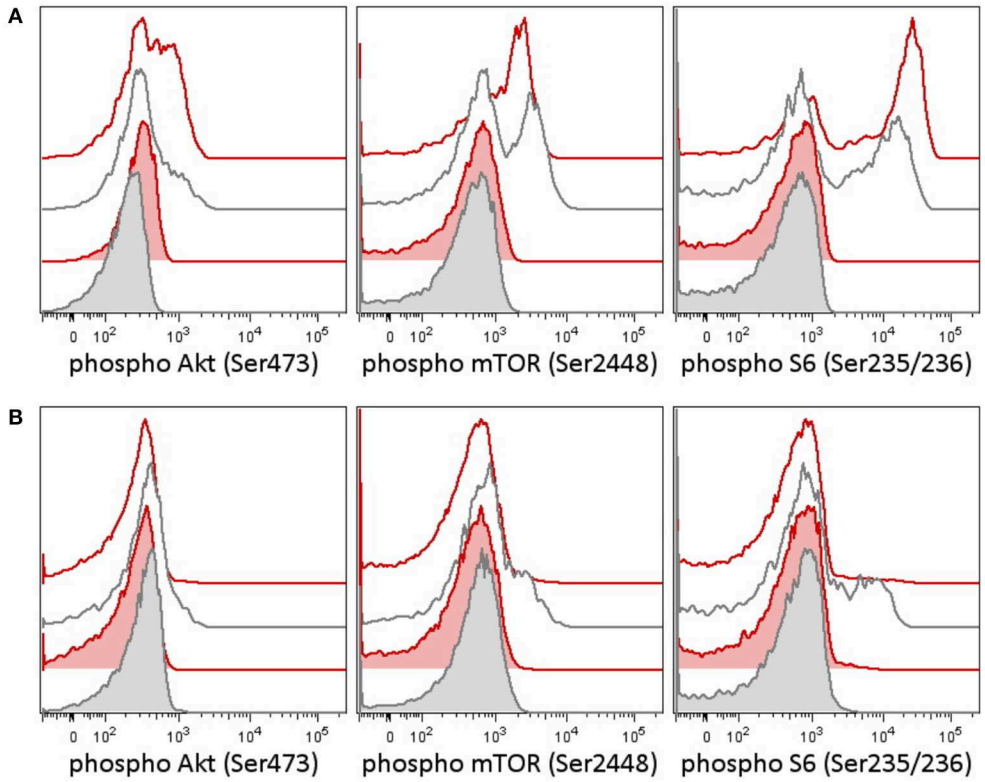

FIGURE 1 | Phosphorylation patterns in the PI3K pathway in peripheral blood T-lymphocytes before **(A)** and after **(B)** therapy in case 1. Case 2 patient had a germline variant of PIK3CD, which was present in the tumor as well. Peripheral blood T-lymphocytes (patient 1's lymphocytes contained only T cells at the time of testing) were tested for activation of the PI3K signaling pathway [reflected as a phosphorylation of Akt (Ser473), mTOR (Ser2448), and S6 ribosomal protein (Ser235/236)] before and following therapy. **(A)** Patient T-lymphocytes showed increased basal phosphorylation of Akt as well as increased phosphorylation of Akt and S6 upon T-cell receptor (TCR) stimulation before treatment compared to an independent healthy control (the result is representative of three independent tests). **(B)** A week following the addition of idelalisib (a PI3K inhibitor), to the patient's therapy, the phosphorylation of Akt, mTOR, and S6 dropped down. CD3+ T-lymphocytes are shown in basal state (tinted histograms) and 15 min upon anti-CD3/CD28/CD49d stimulation (blank histograms). Red, patient 1; black, healthy control.

the longest PFS this child had achieved. The comparison of his earlier therapies reveals that he had achieved PFS1 6 months on the initial standard BFM protocol, and PFS2 only 1 month on the intensive retrieval therapy using anti-CD20 (obinutuzumab), ICE, and ibrutinib. His individualized therapy was outpatient based, associated with minimal treatment-related toxicities and allowed the child to return to school and perform all activities of daily living.

Case 2

A 3-year-old boy diagnosed abroad with widely disseminated Burkitt lymphoma (abdomen, bone marrow, and both kidneys) was initially treated with the same standard BFM-based chemotherapy, but without rituximab. Before the completion of the fifth cycle, the patient had disease progression with a biopsy-positive new lesion in the right cheek. He continued with a relapse ALL protocol/ALL-REZ BFM 2002 in his home country outside the Czech Republic. As no therapeutic response was achieved, he was referred to our institution for a second opinion and management. He received two cycles of R-ICE (rituximab, ifosfamide, carboplatin, etoposide) given as per the ANHL0121 protocol achieving partial response, but the treatment was accompanied with severe life-threatening toxicities. He underwent surgery to obtain specimen for theranostic testing; however, the amount of the tumor tissue was not sufficient for all molecular studies. Based on our previous success in case 1 and as bridging to high-dose chemotherapy, he therefore continued with ibrutinib 140 mg/m^2 daily, idelalisib 100 mg/m^2 daily, and cyclophosphamide 1.5 mg/kg daily week on/week off for 6 weeks. Due to toxicities of intensive therapies and a clinical need for further therapy as bridging to stem cell transplant, the targeted agents were in this case based on our previous experience and a literature review. Despite a high-dose carmustine, etoposide, cytarabine, melphalan (BEAM) chemotherapy as per the AHOD0121 protocol (13) and autologous stem cell transplant being performed, he continued to do poorly. The patient had disease progression 3 weeks after BEAM conditioning and autologous stem cell transplant with a new lesion in the abdomen and continued to progress with massive L3 blast presence in the cerebrospinal fluid. He died due to disease progression 11 months from the initial diagnosis and 6 months after his first progression.

Case 3

A 12-year-old boy was diagnosed with bulky abdominal Burkitt lymphoma. The patient was initially treated as per the standard BFM B-NHL Registry 2012 protocol with the addition of rituximab, but he achieved only partial response after two cycles, and assessment after four cycles revealed residual tumor with still increased FDG PET avidity. Three months later, the FDG PET/CT showed radiological progression of the primary tumor and dissemination in the right retromandibular area and anterior mediastinum. The relapse of Burkitt lymphoma was confirmed by biopsy. However, WES from the relapsed tumor sample revealed high tumor mutation burden−31 mutations/Mb; moreover, gene expression profiling detected strong expression of PD1, and the overall expression patterns of the case 3 were very similar to case 2 patient with very high fibronectin expression. First, participation in the randomized ibrutinib retrieval trial was planned here; however, based on molecular profiling and our previous experience from case 2, we have prioritized immune therapy here. He achieved radiological partial remission after third R-ICE cycle and then continued with nivolumab single agent only. After 12 weeks of nivolumab, he achieved first complete remission. His first PFS on standard intensive protocol was 7 months, but the second PFS with using immunotherapy is 14 months.

Analyses

Somatic exome analysis of relapse samples revealed variants in the *TP53* gene in cases 1 and 2 (p.R273C in case 1 and p.R248L in case 2, NM_000546). p.R273C and p.R248L in *TP53* have been previously described as loss of function mutations based on *in vitro* functional analyses (14–19). Somatic exome analysis in case 1 detected a number of variants; the selected ones are shown in **Supplementary Material 2**. Germline exome analysis in case 1 also confirmed p.S312C (NM_005026) variant in the *PIK3CD* gene in the heterozygous form. Somatic exomes of cases 2 and 3 revealed a number of variants; the selected ones are also available in **Supplementary Material 2**.

Gene expression profiles of all three cases proved to be very similar; the highest expressions showed genes involved in immune system (*BTK*, *CD79A*, *CD79B*, and *KLHL6*). In cases 1 and 2, increased expression also showed genes involved in DNA damage response (*BRCA1*, *BRCA2*, *FANCA*, and *FANCD2*). In case 1, *CSF1R* and *PDGFRA* genes were also found to be increasingly expressed, while no genes coding tyrosine kinases showed to be overexpressed in case 2. In case 3, increased expressions showed genes involved in fibroblast growth factor signaling. In comparison to other pediatric oncology patients analyzed at our institute, transcriptome analysis in cases 1 and 2 revealed significantly increased expression of the *MYC* proto-oncogene.

In case 1, two samples of the tumor tissue were also analyzed for activity of cell signaling pathways using phosphoprotein arrays for detection of RTKs, MAPKs, serine/threonine kinases, and other signaling protein as specified above: tumor tissue sample after the first line of treatment (**Figure 2**: case 1a) and second sample taken during the treatment of relapsed disease (**Figure 2**: case 1b). Phosphorylation profiles showed high relative activities of EGFR, PDGFRβ, ROR2, CREB, ERK1/2, and HSP27 in both samples. Furthermore, a very high level of phosphorylation was detected for p53 protein on Ser46 in the second sample in comparison to the first sample from this patient. This finding is in full accordance with the previous proapoptotic treatment including etoposide administration (20). In case 2, nevertheless, phospho-RTK analysis (**Figure 2**: case 2) revealed high phosphorylation of EGFR and PDGFRβ, and the phosphorylation profile of MAPKs, serine/threonine kinases, and other signaling proteins showed high activities of CREB, ERK1/2, and HSP27 in ascending order of density value.

Serology of Epstein–Barr virus (EBV) revealed the IgG positivity of EBV nuclear antigen (EBNA)-1 and the IgG positivity of viral capsid antigen (VCA) as well case 1 and case 2.

DISCUSSION

The introduction of highly intensive multiagent chemotherapy has dramatically improved the survival rates of primary childhood Burkitt lymphoma. While the initial treatment can have an over 90% success rate using standard intensive chemotherapy with rituximab, the outcome of children with relapsed Burkitt lymphoma is still very poor. The difficulties with treating chemotherapy-resistant relapsed tumors suggest an evolution of a more complex and more resistant disease (21), as could be documented by a new TP53 mutation in our case 1 at relapse, which was suggested by phosphoproteomic assay as well. The overview of our three cases reveals children with some very similar characteristics of their diseases, with alike pattern of cell signaling in tumor tissue, treated with identical agents in the first part of their relapse treatment, who experienced very dissimilar outcomes after the first relapse. It suggests that the tumors with similar histological features may harbor chemotherapy-resistant, genetically and biologically distinct subclones that become more dominant after intensive chemotherapy (21). At presentation, a fraction of these chemotherapy-resistant subpopulations may be small but, following intensive maximum tolerated dose-based chemotherapy, probably increases, and the tumor residuum is subsequently populated by resistant subclones. This evolution was furthermore evident on the evolution of molecular findings in the first patient and supports the need for a careful theranostic analysis and repeated biopsies whenever clinically indicated. Treatment of relapsed disease should be based on a detailed molecular analysis of the most recent available sample, i.e., at the time of relapse or progression rather than on original tumor biopsy only. The choice of drug combinations reflecting a broader molecular profile was based on reports that customized combinatorial therapies may produce more sustained responses (22, 23). Furthermore, as many biological agents are in fact chemotherapy sensitizers, their proper dosage should carefully be titrated to avoid severe systemic toxicity. In case 1, we have started with a single-agent idelalisib to target what was thought to be the driver mutation and gradually added additional targeted agents but at doses about 50% of those recommended in the Summary of Product Characteristics to avoid severe toxicity.

FIGURE 2 | The relative phosphorylation analysis of tumor tissue samples. Human Phospho-MAPK Array Kit (R&D Systems) was employed for the detection of phosphorylation status of 49 RTKs, 26 MAPKs, serin/threonin kinases, and other signaling proteins, which performed using phosphoprotein arrays.

To successfully apply precision oncology principles into clinical practice, a requisite testing for molecular targets for each patient needs to be completed. As pointed above, while all three patients had histologically identical disease and were given the same combination of agents in the first- and two of them as second-line treatments, in case 2, we did not have a representative tumor sample timely available and his therapy was based only on detailed literature review and not the theranostic concept (24–26). The biology of the relapsed disease of case 3 reflected by transcriptome was similar to that of case 2, so a different approach could be undertaken, and while reflecting high mutational burden and increased expression of the PD-1L detected by immunohistochemistry and transcriptome, anti-PD-1 antibody was successfully used here.

While analyzing the transcriptomic results including considerations of gene and network interactions using https://string-db.org/ and http://www.genome.jp/kegg/pathway.html databases (6, 21), we were able to distinguish different patterns of tumor biology among our patients. Case 1 suggested neurotrophic receptor tyrosine kinase 1 (NTRK1) as a signaling protein and one of the best targets. In case 2 and case 3, in contrast, despite being clinically and histologically similar, transcriptomic results suggest an entirely different network, where fibronectin 1 (FN1) has a very complex downstream impact. Because FN1 is not a signaling protein and a druggable target, it is likely that we missed the putatively most important pathway in case 2. One may speculate that integrin inhibitors like cilengitide could be a better therapeutic option here. For case 3, FN1 seemed to be the key molecular hub as well, and it was one of the reasons for clinical decision to rely on tumor mutational burden and PD-1 ligand expression and treat the patient with immune therapies, rather than small molecules.

The localization of *MYC* proto-oncogene on q24 of the human chromosome 8 and its translocation to chromosome 14 is considered pathogenic in most cases of Burkitt lymphoma. In our cases, the RNA transcription analyses as described above indicate the activations of different sets of genes. These patients were almost identical in their clinical presentation, histology, *MYC* status, and initial clinical response to standard chemotherapy. Early clinical testing initiatives are beginning to employ individual profiles/fingerprint analyses to compile patients into histologically or biologically similar series (27), and as these efforts continue, new clinical trial designs will emerge (28, 29).

The research that has emerged over the last 40 years disproves the concept that cancer is a consequence of a single oncogenic change. It is widely accepted that an initiating oncogenic change such as translocation involving MYC is interpreted within the patient's genome, and further genomic alterations lead to the oncogenic inducers hijacking host-specific physiological responses such as angiogenesis, inflammation, and immune evasion. These normal physiological responses are not detected by DNA mutational analysis because they represent reactivation of developmentally silent pathways. We advocate the use of combinations of biological agents addressing not only the DNA mutations but also the normal physiological responses of the host as they are reflected in the individual's molecular signature reflected on transcriptomic and proteomic levels. In case 3, we successfully used immunotherapy reflecting the molecular profile of the tumor. In cases 1 and 2, we used a combination of ibrutinib (inhibitor of BCR signaling), idelalisib (direct PI3Kdelta inhibitor), valproate (HDAC inhibitor with potential to enhance responsiveness to immune therapies), and nivolumab (a host immune response modulator). Both patients were intended to receive an immune-supportive therapy using autologous dendritic cell vaccination with non-immune-suppressive maintenance agents such as checkpoint inhibitors, but only case 1 patient had achieved sufficient duration of the clinical response to live long enough to enable the preparation of his vaccine. Unfortunately, because we did not have the benefit of molecular information on genome or transcriptome in case 2, the therapy could not be customized enough to provide a more effective therapeutic combination. Our results revealing highly phosphorylated EGFR, PDGFRβ, ROR2, ERK1/2, or Hsp27 in all samples are also in accordance with previously published findings on Burkitt lymphoma (30, 31). Interestingly, activation of EGFR and ERK signaling via EBV oncoprotein LMP1 was also reported (32, 33) and our results thus concur with the latent EBV infection as suggested by serological analysis.

One of the most interesting observations was the discordance between laboratory and clinical responses to biomarker-based targeted therapy in case 1. Even though there was evidence of normalization of PI3K pathway activity, the evidence of radiological response was significantly delayed and gave an impression that the patient continued to progress. As has been frequently observed with biological therapies, the biomarker response may be more informative and preceded in this case the radiological response. While using biological therapies, we must allow sufficient time to pass before the patient is evaluated using present radiomorphological methods.

As we show, in cases where individualization of treatment protocols can be based on the recent molecular information, the likelihood of successful therapy may be increased, but the use of a targeted agent without laboratory evidence of contemporary target activation may not only lack benefit—it may even be harmful. Similarly, while treating sepsis, we are not using several-month-old microbiology results to guide antimicrobial treatment. Considering that there are presently numerous initiatives intending to study the addition of idelalisib and/or ibrutinib to existing retrieval therapies for relapsed and refractory mature B-cell lymphomas, it may be of value to collect enough samples for tumor tissue analysis and enable similar retrospective comparisons of patients who either failed or responded to therapy. An attractive concept inspired by our cases may be the successful sequence of different treatment modalities, such as intensive chemotherapy to debulk the initial tumor volume, followed by targeted biomarker-based treatment and stimulation of autologous immune response later on to consolidate the response.

CONCLUSION

Precision medicine has significantly altered the practice of clinical oncology, but no standardized approach to the choice of these therapies exists. The three cases presented here emphasize that despite similarities in the presentation, histology, age, tumor site, and initial treatment response, the biology of tumors may differ significantly between cases and may change over time. Case 2 patient had an entirely different molecular signature and thus biology, without underlying relevant germline mutation, but such differences in molecular profile could be appreciated in retrospect only. We conclude that considering the dire outcomes of relapsed Burkitt lymphoma, theranostic testing may identify the most frequent molecular profiles that lead to therapeutic resistance and may help to improve frontline therapies sufficiently to prevent relapses and 1 day to replace our decade-old and toxic drugs like anthracyclines and alkylating agents.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available because it is not in accordance with our institutional policy. We handle data of rare entities that may be at risk of identification. Requests to access the datasets should be directed to Kristyna Polaskova, polaskova.kristyna@fnbrno.cz.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee for Multicenter Clinical Trials of the University Hospital Brno. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KP wrote the draft of the manuscript and evaluated patient record. TM wrote the manuscript. DZ and AM evaluated patient records. MK did the statistical analyses. PMa, ZK, and PMu participated on the treatment decision and evaluated patient records. MJ performed pathological investigation. JT performed surgical procedures. JS and IC performed the radiological evaluations. DV, LZ-D, and SK participated on the manuscript. HN, KP, OS, PF, JN, RV, VK, OH, and TF performed biological samples analyses. GK supervised and wrote the manuscript. JS conceived and supervised the project and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01531/full#supplementary-material

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Assessment of Tumor Mutational Burden in Pediatric Tumors by Real-Life Whole-Exome Sequencing and In Silico Simulation of Targeted Gene Panels: How the Choice of Method Could Affect the Clinical Decision?

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Abstract: Background: Tumor mutational burden (TMB) is an emerging genomic biomarker in cancer that has been associated with improved response to immune checkpoint inhibitors (ICIs) in adult cancers. It was described that variability in TMB assessment is introduced by different laboratory techniques and various settings of bioinformatic pipelines. In pediatric oncology, no study has been published describing this variability so far. Methods: In our study, we performed whole exome sequencing (WES, both germline and somatic) and calculated TMB in 106 patients with high-risk/recurrent pediatric solid tumors of 28 distinct cancer types. Subsequently, we used WES data for TMB calculation using an in silico approach simulating two The Food and Drug Administration (FDA)-approved/authorized comprehensive genomic panels for cancer. Results: We describe a strong correlation between WES-based and panel-based TMBs; however, we show that this high correlation is significantly affected by inclusion of only a few hypermutated cases. In the series of nine cases, we determined TMB in two sequentially collected tumor tissue specimens and observed an increase in TMB along with tumor progression. Furthermore, we evaluated the extent to which potential ICI indication could be affected by variability in techniques and bioinformatic pipelines used for TMB assessment. We confirmed that this technological variability could significantly affect ICI indication in pediatric cancer patients; however, this significance decreases with the increasing cut-off values. Conclusions: For the first time in pediatric oncology, we assessed the reliability of TMB estimation across multiple pediatric cancer types using real-life WES and in silico analysis of two major targeted gene panels and confirmed a significant technological variability to be introduced by different laboratory techniques and various settings of bioinformatic pipelines.

Keywords: pediatric tumors; tumor mutational burden; TMB; whole-exome sequencing; gene panel sequencing; immune checkpoint inhibitors

1. Introduction

The cancer cell genome acquires genetic alterations differing from the germline of the host [1]. Somatic mutation rates can be affected by exposure to exogenous factors, such as ultraviolet light or tobacco smoke [2], or by compounding genetic defects, such as DNA mismatch repair deficiency, microsatellite instability, or replicative DNA polymerase mutations [1–3]. These somatic genetic alterations induce and drive carcinogenesis. The type and the number of acquired mutations varies among the cancer types but also among the affected individuals [4]. Some of these mutations lead to the formation of tumor-specific neoantigens, which could be recognized by a patient's immune system as non-self and which are highly clinically relevant since these neoantigens can make the cancer cells sensitive to treatment with immune checkpoint inhibitors (ICIs) against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) in various cancers including melanoma [5], non–small-cell lung cancer (NSCLC) [6], kidney cancer [7], bladder cancer [8] and others [9]. The genomic landscape of smoking-induced NSCLC and UV light-induced melanoma is often characterized by a high number of acquired alterations, while leukemias and pediatric tumors show the lowest mutations counts.

Rapidly developing genomic methods based on next-generation sequencing (NGS) simplified the detection and quantification of these acquired changes on the level of individual cancer genomes. Tumor mutational burden (TMB) is a quantitative measure of acquired somatic mutations in the cancer cell genome. Initial exploratory analyses of TMB in cancer patients [10,11] were carried out using whole exome sequencing (WES). WES is a comprehensive research tool for assessment of genomic alterations across the entire coding region of the ~22,000 genes in the human genome, comprising of 1–2% of the genome [3,12]. Currently, WES-derived TMB values are considered to be the gold standard, but the high cost and long turnaround time limit routine diagnostic applicability of WES. Therefore, targeted NGS cancer gene panels have been promoted for TMB estimation as a feasible and cheaper alternative to WES [13]. Whereas TMB assessed by WES is typically reported as the total number of mutations per cancer cell exome, TMB assessed by gene panel assays is usually referred to as mutations per megabase (mut/Mb) because it differs in the number of genes and target region size [2,3,14]. The precise calculation of TMB may, however, vary depending on the region of tumor genome sequenced, types of mutations included, methods of subtracting germline variants and other aspects of bioinformatic analysis pipeline of the sequencing data [3,15]. Both the FDA-approved FoundationOne CDx (F1CDx) panel and the FDA-authorized Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) panel used correlation between panel- and WES-based TMB to validate the reliability of panel based TMB estimation, and they claimed that these panels can assess TMB accurately (R = 0.74 for F1CDx and R = 0.76 for MSK-IMPACT) [2,13,16]. However, as Wu et al. [13] proposed in their recent work, the overall correlation between the panel- and WES-based TMB could be substantially distorted by outliers (i.e., cases with relatively ultra-high TMB within each cancer type) [13], which might lead to overestimation of the reliability of panel-based TMB estimation. Therefore, additional studies are needed to evaluate the significance of correlation between the WES-based and targeted panel-based TMB values.

As already mentioned, TMB is considered to be a proxy for cancer cell neo-antigenicity and therefore could potentially serve as a predictive biomarker of therapeutic response to ICI. Several studies, especially in NSCLC, retrospectively employed WES or larger NGS panels to determine TMB as a potential response predictor [17–19]. Unfortunately, the definition of cut-off values to separate "high TMB" from "low TMB" tumors is not consistent in recent NSCLC trials. For example, in the CheckMate (CM) trials CM012 (nivolumab and ipilimumab) [20], CM227 (nivolumab and

ipilimumab) [17] and CM026 (nivolumab only) [21] cut-points of 158 mutations, 199 mutations and 243 somatic missense mutations (number of mutations estimated from a commercial gene panel based cut point of 10 mutations per Mbp) were used, respectively [22].

This is the first study in pediatric oncology that aims to assess the reliability of TMB estimation using real-life WES across multiple cancer types and in silico analysis of two major gene panels, which are widely used for routine diagnostics in clinical practice, where various settings of bioinformatic pipeline were employed. The performance and correlation of WES and panel-based TMB assessment methods were evaluated together with potential consequences for clinical decision making where various cut-offs for ICI indication were used.

2. Results

2.1. Comparison of TMB between Real-Life WES and In Silico Targeted Gene Panels

We successfully performed germline and somatic WES and calculated TMB in 106 pediatric patients of 28 distinct cancer types. We stratified patients based on their diagnosis and expressed TMB for each group of patients as a median (min–max) or as a concrete value in cases where there was only one patient within a group (summarized in Table 1). WES-based TMB for each tumor is depicted in Figure 1. The median TMB ranged widely among diagnoses, from 0.3 mutations/Mb in myeloid sarcoma to 14.2 mutations/Mb in Burkitt lymphoma.

Table 1. Comparison of TMB determined by real-life WES and in silico targeted gene panels.

Diagnosis	TMB WES—M1 * Real-Life (Median/Value)	(Min–Max)	TMB MSK—M1 * In Silico (Median/Value)	(Min–Max)	TMB F1CDx—M2 ** In Silico (Median/Value)	(Min–Max)
HGG glioma H3K27M+	2.9	(1.6–15.7)	4.7	(2.6–17.9)	4.5	(2.6–31)
Rhabdomyosarcoma	3.6	(1.7–6.4)	2.6	(1.7–4.3)	2.6	(0–5.2)
Ewing sarcoma	3.1	(0.2–5.1)	2.6	(0–5.1)	2.6	(0–7.8)
Ependymoma	3.1	(1.3–10.4)	1.7	(0–5.1)	3.2	(1.3–9)
Neuroblastoma	3.8	(1.6–17.2)	3.0	(0.9–7.7)	4.5	(1.3–15.5)
Soft tissue sarcoma	3.6	(1.7–6.7)	3.4	(0–6.8)	3.2	(0–9)
Low-grade glioma	3.5	(1.6–6.8)	2.1	(0.9–4.3)	3.9	(1.3–5.2)
High-grade glioma H3K27M wt	4.5	(1.4–269.8)	3.4	(0.9–294.7)	5.2	(1.3–410.9)
Osteosarcoma	2.2	(1.9–7.5)	3.4	(0–5.1)	5.2	(1.3–6.5)
Burkitt lymphoma	14.2	(6.1–100.7)	19.6	(6.8–46.1)	27.1	(6.5–89.2)
Medulloblastoma	3.8	(3.5–63.6)	3.4	(0.9–61.5)	3.9	(1.3–89.2)
Fibromatosis	6.2	(1.1–56.2)	5.1	(1.7–29)	10.3	(1.3–82.7)
Wilms tumor	3.1	(2.3–3.9)	3.4	(2.6–4.3)	2.6	(1.3–3.9)
Renal cell carcinoma	1.8	(1.5–2.1)	4.3	(2.6–6.0)	4.5	(1.3–7.8)
Adrenocortical carcinoma	0.9	-	0.9	-	1.3	-
Plexus choroideus carcinoma	5.2	-	2.6	-	5.2	-
Hepatocellular carcinoma	3.6	-	0.9	-	3.9	-
Disseminated adenocarcinoma	2.3	-	4.3	-	6.5	-
Familiar infantile myofibromatosis	2.1	-	1.7	-	0.0	-
Myeloid sarcoma	0.3	-	0.0	-	0.0	-
Undifferentiated embryonal tumor of spinal canal	3.1	-	2.6	-	2.6	-
Nongerminomatous Germ Cell tumor CNS	2.3	-	1.7	-	1.3	-
Epithelial hepatoblastoma	0.5	-	0.0	-	0.0	-
Spindle cell hemangioma	2.1	-	0.9	-	2.6	-

Table 1. *Cont.*

Fibrodysplasia ossificans progressiva	3.1	-	2.6	-	2.6	-
Hepatosplenic T-lymphoma	0.4	-	0.9	-	0.0	-
Multisystemic Langerhans cell histiocytosis	3.1	-	2.6	-	3.9	-
Gastrointestinal stromal tumor	2.7	-	3.4	-	6.5	-

* M1—Method 1 for calculation of TMB excluding synonymous variants and indels; ** M2—Method 2 for calculation of TMB including synonymous variants and indels.

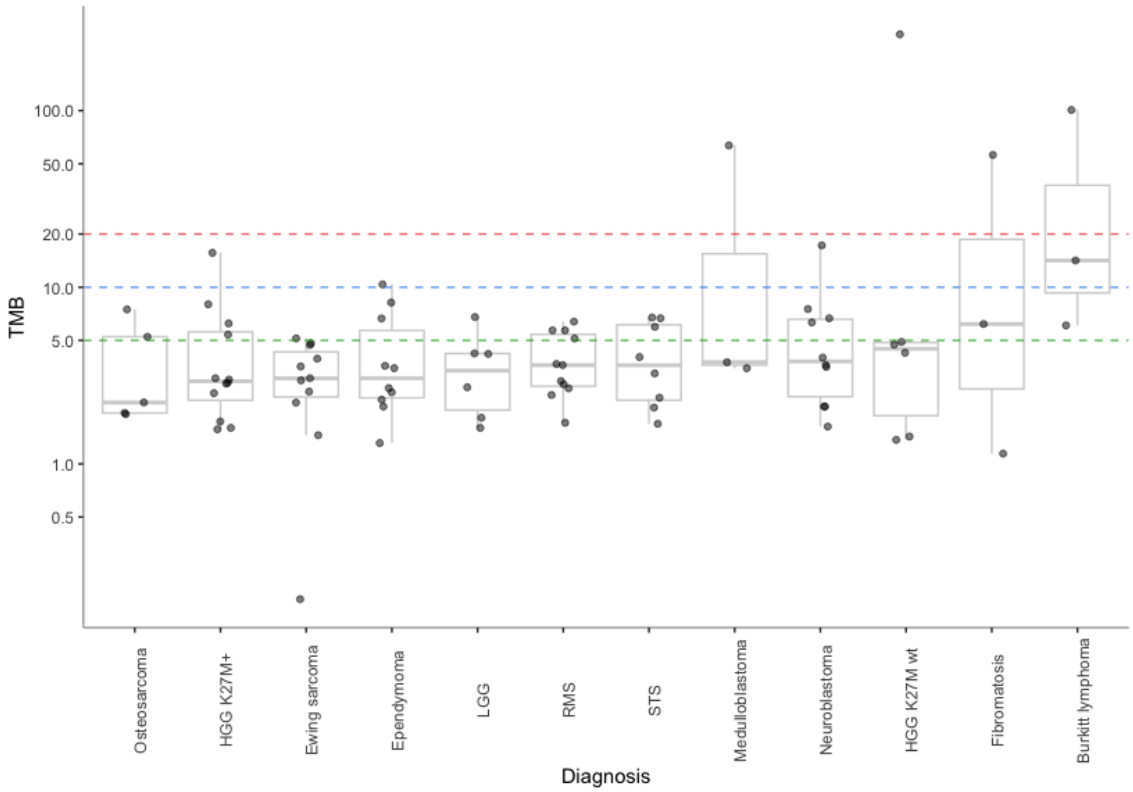

Figure 1. Tumor mutational burden (TMB) values determined in our pediatric cancer patient cohort (WES—Method1) stratified by cancer type. Hypothetical TMB cut-off values are shown as dashed lines (green, TMB ≥ 5; blue, TMB ≥ 10, red, TMB ≥ 20).

Furthermore, we determined, by an in silico approach, whether TMB, as measured by WES, correlates with TMB calculated by the gene sets and bioinformatic approaches used by two commercially available targeted gene panels. Panel-based TMB (MSK-IMPACT and F1CDx) for each group of patients expressed as a median (min–max) or as a concrete value in cases where there was only one patient in a group are summarized in Table 2. We confirmed a strong Pearson correlation of the panel TMB with the WES-based TMB characterized by R = 0.993 (F1CDx), and R = 0.974 (MSK-IMPACT), respectively (Figure 2A,C). Correlation between MSK-IMPACT and F1CDx panels was R = 0.993 (Figure 2B). The TMB assessment method was adapted for each panel accordingly (MSK-IMPACT—Method 1; F1CDx—Method 2). However, when the few hypermutated cases were excluded and only samples with TMB <10 mut/Mb were considered for analysis, the correlation decreased significantly: R = 0.514 (F1CDx), and R = 0.560 (MSK-IMPACT). Correlation between TMBs determined by the two panels remained remarkably higher (R = 0.726).

Table 2. Comparison of TMB determined by real-life WES and the FMI laboratory testing service FoundationOne Heme (F1Heme).

Gender	Age at Diagnosis	Diagnosis	TMB F1Heme Real-Life (Mut/Mb)	TMB WES—M1 * Real-Life (Mut/Mb)	Same Sample (Yes/No)
F	9	Renal cell carcinoma	1.63	1.45	yes
F	7	Diffuse intrinsic pontine glioma H3K27M+	2.44	1.60	yes
M	13	Desmoid fibromatosis	0.81	1.14	yes
M	6	Spindle cell hemangioma	0.81	2.05	yes
F	14	Gastrointestinal stromal tumor	4.07	2.71	yes
F	14	Osteosarcoma	2.44	1.91	yes
M	2	Langerhans cell histiocytosis	2.44	3.11	yes
M	11	Wilms tumor	1.63	2.34	yes
M	11	Ewing sarcoma	1.63	2.57	yes
F	7	Ependymoma	2.44	3.48	yes
M	18	Embryonal rhabdomyosarcoma	4.89	2.82	yes
F	14	Ewing sarcoma	1.63	3.57	yes
F	6	Wilms tumor	0.81	3.91	yes
F	18	Ewing sarcoma	0.81	2.97	yes
M	9	Alveolar rhabdomyosarcoma	3.26	3.62	yes
F	5	Diffuse intrinsic pontine glioma	2.44	2.85	yes
M	10	Ewing sarcoma	1.63	0.17	yes
F	1	Neuroblastoma	1.63	7.53	yes
F	10	Ewing sarcoma	7.33	4.82	yes
M	20	Glioblastoma H3G34R+	7.33	8.02	yes
F	2	Neuroblastoma	5.70	6.33	yes
F	1	Embryonal rhabdomyosarcoma	1.63	6.39	yes
M	3	Burkitt lymphoma	10.59	6.08	yes
M	7	Burkitt lymphoma	19.55	14.18	yes
M	18	Glioblastoma	265.56	269.75	yes
F	10	Low-grade astroblastoma	1.63	1.83	no
M	4	Adrenocortical carcinoma	0.00	0.88	no
M	15	Hepatocellular carcinoma	2.44	3.59	no
M	3	Epithelial hepatoblastoma	2.44	0.46	no
M	5	Embryonal rhabdomyosarcoma	6.52	3.68	no
M	3	Embryonal rhabdomyosarcoma	4.07	5.71	no
F	7	Glioblastoma	0.81	4.48	no
M	1	Anaplastic ependymoma	1.63	6.65	no
F	4	Diffuse intrinsic pontine glioma H3K27M+	9.78	5.39	no

* M1—Method 1 for calculation of TMB excluding synonymous variants and indels.

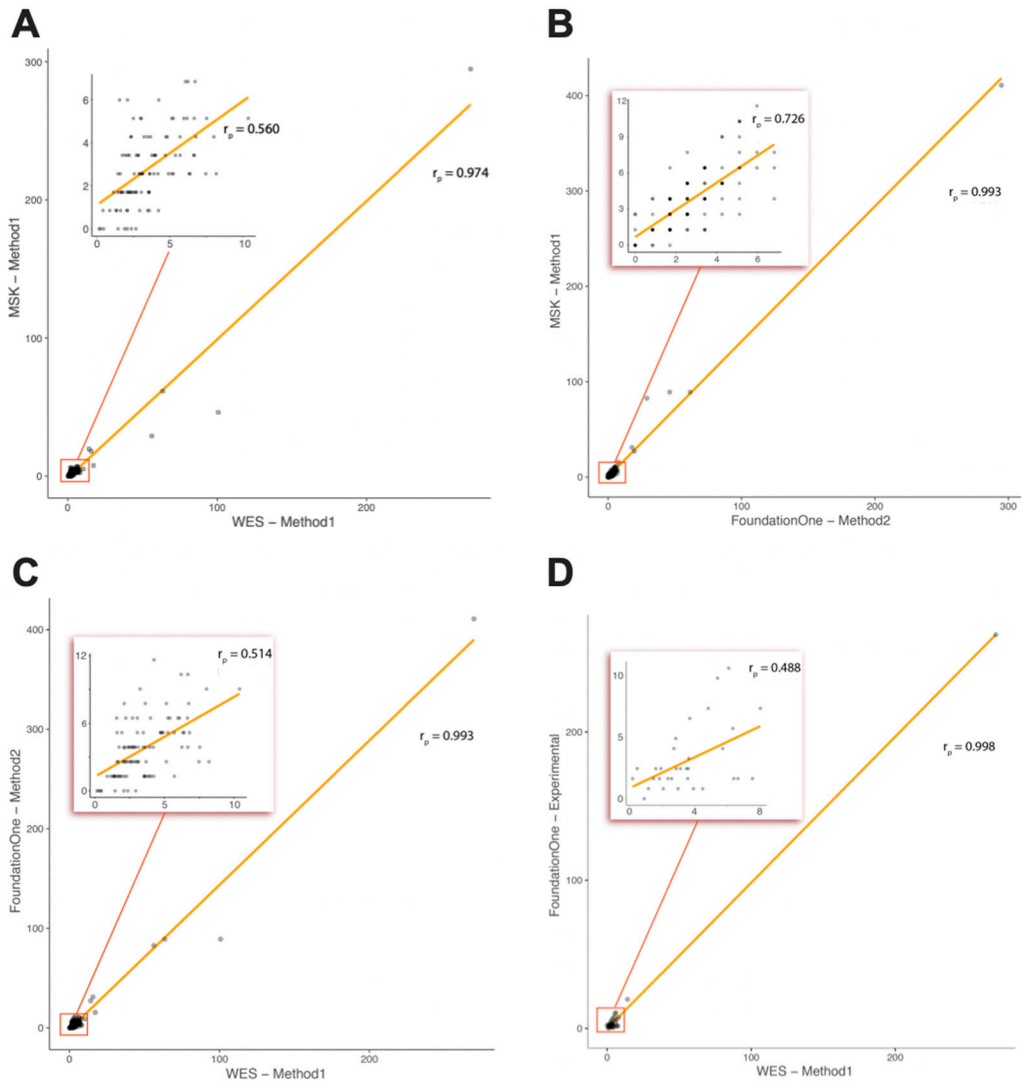

Figure 2. Correlation of tumor mutational burden (TMB) determined by real-life WES and targeted gene panels: real-life WES vs. in silico MSK-IMPACT (**A**), in silico F1CDx vs. MSK-IMPACT (**B**), real-life WES vs. in silico F1CDx (**C**), real-life WES vs. real-life laboratory service F1Heme (**D**).

2.2. Comparison of TMB between Real-Life WES and the Foundation Medicine Inc. (FMI) Testing Service (Subcohort of Patients)

In the subgroup of 34 patients (randomly selected from the patients where a Formalin-Fixed Paraffin-Embedded (FFPE) block with tumor tissue was available), comparative study of real-life WES-based TMB assessment and the FMI testing service was performed. For the WES samples, tumor and normal tissue were each sequenced in order to distinguish germline polymorphisms from somatic mutations. For the targeted FMI testing, no matched normal material was sequenced; rather, genomic variants were stringently filtered to eliminate germline polymorphisms, as declared by the vendor. For TMB determination from WES data, we used Method 1 (excluding indels and synonymous mutations). The FMI testing services are done using Method 2 (including indels and synonymous mutations). In nine cases, different samples from one resection or biopsy collection were used. This is summarized in Table 2. However, the Pearson correlation between TMBs determined by these two real-life approaches was comparable to the correlation of real-life WES and in silico F1CDx panel (R = 0.998 vs. R = 0.993) indicating the relevance of the in silico approach for TMB assessment comparative studies. When hypermutated cases were excluded, correlation decreased to R = 0.488 (Figure 2D), which is similar to the decrease observed in the in silico approach (R = 0.514).

2.3. WES-Based TMB Values during Tumor Progression

In nine cases, we determined the TMB by WES in sequential tumor biopsies or tumor tissues from surgical resection. In five cases, we used tumor tissue from a primary tumor and its relapse. In the remaining four cases, tumor tissue was collected from two consequent local or metastatic relapses. TMB values are summarized in Table 3. In seven out of nine cases, an increase in TMB in the second tumor tissue was observed, with the average increase being 1.6 ± 1.3 mut/Mb.

Table 3. WES-based TMB values during tumor progression in nine patient case cohorts.

Gender	Age at Diagnosis	Diagnosis	Diagnosis/Relapse	Year of Biopsy	TMB (WES M1 *) Real-Life
F	9	Supratentorial ependymoma	local relapse	2016	2.31
			local relapse	2018	3.88
F	1	Neuroblastoma	metastatic relapse	2017	7.53
			metastatic relapse	2018	3.17
M	11	Ewing sarcoma	primary tumor	2017	2.57
			local relapse	2018	4.19
M	5	DIPG	primary tumor	2015	2.51
			local relapse	2018	6.68
F	10	LG astroblastoma	primary tumor	2017	1.83
			local relapse	2018	3.05
M	3	Epithelial hepatoblastoma	primary tumor	2016	0.46
			local relapse	2018	2.48
F	2	Ependymoma	primary tumor	2014	10.38
			metastatic relapse	2018	10.53
M	18	Osteosarcoma	metastatic relapse	2018	7.47
			metastatic relapse	2018	8.10
M	1	Infantile myofibromatosis	metastatic relapse	2015	2.08
			metastatic relapse	2018	1.88

* M1—Method 1 for calculation of TMB excluding synonymous variants and indels.

2.4. Consequence of TMB Assessment Method for ICI Indication

TMB as a predictive biomarker is currently the focus of several clinical trials with ICI. We have evaluated how the sequencing region (WES vs. the gene set used in MSK-IMPACT vs. the gene set used in F1CDx) and method for TMB calculation affect the final TMB and potential ICI indication when various hypothetical cut-off values are applied. Results of this analysis are summarized in Table 4. As expected, the number of patients above a cut-off is always higher with WES-based TMB assessment (compared to panel-based) and when TMB is assessed by Method 2 (including indels and synonymous mutations). Number of patients above a cut-off differs significantly when low TMB cut-off value is applied (cut-off ≥ 5). With the increasing cut-off values, the significance of technological variability introduced by sequencing various genome regions and different TMB calculating methods decreases. However, even with a relatively high cut-off value (cut-off ≥ 20), the number of pediatric patients hypothetically indicated for ICI therapy differs between TMB groups calculated with Method 1 and Method 2 (e.g., four vs. seven pediatric patients with WES).

Table 4. WES-based TMB values during tumor progression in nine patient case cohorts.

	TMB—M1 * In Silico (Number of Cases Above Cut-Off)			TMB—M2 ** In Silico (Number of Cases Above Cut-Off)		
Cut-off for ICIs Indication (mut/Mb)	≥5	≥10	≥20	≥5	≥10	≥20
WES	30	8	4	75	25	7
MSK-IMPACT	23	6	4	61	12	6
F1CDx	24	7	5	42	11	6

* M1—Method 1 for calculation of TMB excluding synonymous variants and indels; ** M2—Method 2 for calculation of TMB including synonymous variants and indels; ICIs—immune checkpoint inhibitors.

3. Discussion

The predictive power of TMB as a biomarker for response to ICI is currently being investigated in many clinical trials across various cancer types. Patients with a higher TMB are more likely to respond to ICI in various settings, including PD-(L)1 blockade in NSCLC [10], CTLA-4 blockade in malignant melanoma [11], and combined PD(L)-1 and CTLA-4 blockade in NSCLC [17]. Studies have shown that TMB is to a large extent independent of the PD-L1 status and might thereby identify additional subgroups of patients who benefit from ICI [17,20,22].

Based on these clinical observations, TMB became an emerging predictive biomarker for ICI in various cancer types, and an urgent need occurred to answer the questions concerning the technological aspects affecting TMB detection by WES and targeted panel sequencing to ensure implementation of lab developed tests that guarantee optimal reference standard quality for patient stratification [19].

In initial studies, WES was widely used to determine TMB and is still considered to be the gold standard; however, targeted sequencing panels are more readily interpretable and are a more pragmatic and potentially cost-effective approach to TMB testing in clinical diagnostics [3]. While in the context of clinical trial, TMB testing is mainly carried out by commercial vendors, many clinical laboratories depending on the regulatory approval context may eventually use in-house designed panels to determine TMB scores [22]. Endris and others have already investigated the minimum required size of a gene panel by comprehensive in silico analyses of available WES data sets and have shown that at least 1 Mbp of exonic and/or intronic region should be sequenced to achieve a similar power in discriminating ICI responders from non-responders comparable to WES [19]. Furthermore, Buchhalter at al. showed that "size does matter", with an optimal panel size being between 1.5 and 3 Mbp, considering the benefit–cost ratio, and that the inclusion of all point mutations (instead of only missense mutations) in the TMB calculation is possible and recommendable to enhance precision [9].

In our study, we focused on the potential technological variability introduced to TMB scoring by the usage of various platforms and bioinformatic pipelines for their assessment in pediatric tumors. As a reference method, we performed WES and subsequently in silico simulated two most frequently used sequencing panels, MSK-IMPACT and F1CDx. We confirmed a strong Pearson correlation of the panel-based TMB with the WES-based TMB; however, when the few hypermutated cases were excluded and only samples with TMB < 10 mut/Mb were considered for analysis, the correlation decreased significantly (Figure 2). This indicates a significant bias introduced to correlation analysis by only a few hypermutated cases included in the study. Correlation between samples with TMB < 10 mut/Mb was not satisfactory and probably lead to significant clinical misclassifications in the routine diagnostic scenario based on the usage of a cut-off value in the range of 5 to 15 mut/Mb. Similar observations were also provided by other authors describing adult tumors [9,19].

In a subgroup of patients, we performed a comparative study of real-life WES-based TMB assessment and the FMI testing service where we observed a similar effect of the hypermutated cases on the correlation significance. In agreement with others [9,19], we observed that the identification of high TMB tumors can be reliably achieved by any of the tested methods (cases with ultra-hypermutated tumors). However, the vast majority of tumors have intermediate TMB values; in these cases,

a technological variability interferes with the reliable differentiation between TMB-high and low tumors [9,19].

In nine cases, we determined the TMB by WES in sequential tumor biopsies or tumor tissues from surgical resection. As expected, in seven out of nine cases, there was an increase in TMB in the second tumor with the average increase being approx. 2 mut/Mb. Surprisingly, in two cases, we observed a decrease in TMB, which could be explained mainly by the quality of the tumor tissue specimen and a low content of tumor cells in the second tumor which could decrease detectable mutations used for TMB assessment. It is important to mention that tumor content in the tissue specimens is an important factor affecting TMB scoring and is often not considered in TMB studies.

Finally, we evaluated how the sequencing region (WES vs. the gene set used in MSK-IMPACT vs. the gene set used in F1CDx) and the bioinformatic pipeline used for TMB calculation affect the final TMB and potential ICI indication when various hypothetical cut-off values are applied. In general, as expected, the number of patients above a cut-off is always higher in WES-based TMB assessment (compared to panel-based) and when the TMB is assessed by Method 2 (including indels and synonymous mutations). We also found that with the increasing cut-off values, the significance of technological variability and consequent clinical misclassification decreases. However, certain combinations of settings of TMB assessment methods (e.g., WES-M2 vs. F1CDx-M1), compounded by the use of a cut-off value of 10 mut/Mb, yield extremely different results. While the first approach predicts 25 patients to be good responders to ICI, the second approach predicts only seven patients. This indicates a potentially very strong misclassification issue for routine diagnostics. Based on the currently available results from clinical trials, it is very difficult to judge whether TMB assessed by Method 1 or Method 2 is a more accurate predictive biomarker of response to ICI therapy. Unfortunately, this in silico modeling has not been performed in the context of clinical outcomes from ICI trials.

4. Materials and Methods

4.1. Patients and Biological Specimens

We reviewed tumor mutational burden (TMB) results from 106 patients with pediatric high-risk/recurrent solid tumors (both newly diagnosed and relapsed) who had undergone laboratory WES at Central European Institute of Technology (CEITEC, Masaryk University, Brno, Czech Republic). Informed consent was obtained from all patients and all experiments using clinical samples were performed in accordance with the approved international guidelines. After surgical resection of the tumor or collection of the tumor biopsies, tissue samples were evaluated by an experienced surgical pathologist for the tumor cell content, and only specimens with more than 20% of the tumor cells were included. In addition, peripheral blood was collected to obtain DNA for germline WES. Number of patients stratified according to their diagnoses and related clinical data are summarized in Table 5. In nine cases, we collected two consequent tissue specimens (diagnosis/relapse or two relapses) and both were used for WES and TMB assessment.

4.2. DNA Isolation

Tumor DNA was extracted from the FFPE samples or fresh frozen tissues using QIAmp DNA FFPE Tissue Kit (Qiagen, Venlo, The Netherland) or QIAamp DNA Micro Kit (Qiagen). Germline DNA was extracted from peripheral blood leukocytes using QIAamp DNA Micro Kit (Qiagen). The purified DNA was quantified using Qubit 2.0 Fluorometer and NanoDrop 2000c spectrophotometer (both Thermo Fisher Scientific, MA, USA).

Table 5. Number of patients stratified according to their diagnoses and baseline clinical data.

Diagnosis	Number of Patients	Gender Ratio (F/M)	Age Median	Age (Min–Max)	Type of Sample Ratio (Primary Tumor/Local or Metastatic Relapse)
High-grade glioma H3K27M+	12	8/2	9	4–20	12/0
Rhabdomyosarcoma	11	7/4	5	0–18	6/5
Ewing sarcoma	11	6/5	11	8–18	2/9
Neuroblastoma	10	6/4	2	1–8	1/9
Ependymoma	10	6/4	5.5	1–16	4/6
Non-rhabdomyosarcoma soft-tissue sarcomas	8	2/6	12	8–19	0/8
High-grade glioma H3K27M wt	6	0/6	16	8–23	5/1
Low-grade glioma	6	1/5	9.5	3–19	1/5
Osteosarcoma	5	4/1	18	14–28	0/5
Burkitt lymphoma	3	0/3	7	3–12	0/3
Medulloblastoma	3	0/3	4	2–5	1/2
Fibromatosis	3	1/2	17	13–20	1/2
Wilms tumor	2	1/1	8.5	6–11	1/1
Renal cell carcinoma	2	1/1	13.5	9–18	1/0
Adrenocortical carcinoma	1	F	4	-	primary tumor
Choroid plexus carcinoma	1	M	1	-	primary tumor
Hepatocellular carcinoma	1	M	15	-	primary tumor
Lung adenocarcinoma	1	F	15	-	metastatic relapse
Familiar infantile myofibromatosis	1	M	1	-	primary tumor
Myeloid sarcoma	1	F	5	-	primary tumor
Undifferentiated embryonal tumor of spinal canal	1	M	2	-	primary tumor
CNS germ cell tumor	1	M	11	-	local relapse
Epithelial hepatoblastoma	1	M	3	-	primary tumor
Spindle cell hemangioendothelioma	1	M	6	-	primary vascular malformation
Fibrodysplasia ossificans progressiva	1	F	1	-	primary tumor
Hepatosplenic T-lymphoma	1	M	17	-	diagnostic aspiration/bone marrow
Multiple system Langerhans cell histiocytosis	1	M	2	-	metastasis
Gastrointestinal stromal tumor	1	F	14	-	metastatic relapse

4.3. Whole Exome Sequencing

Libraries for whole exome capture and sequencing were prepared using TruSeq Exome Kit (Illumina, CA, USA) according to manufacturer´s recommendations. Quantity and quality of the exome libraries were checked using Qubit 2.0 Fluorometer and NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). Prepared libraries were loaded onto NextSeq 500/550 Mid Output Kit (150 cycles) and sequenced on the NextSeq 500 instrument (both Illumina). Sequencing coverage for both exomes was >20 × at >90% of captured regions.

4.4. Bioinformatic Analysis

Sequencing reads in FASTQ format were mapped to the human reference genome hg19 with the BWA-MEM algorithm [23] for both the tumor and the healthy control sample. The resulting alignments in BAM format were postprocessed with the SAMBLASTER program [24] for marking PCR duplicates. The final alignment file of the control sample was used to assess single nucleotide variants (SNVs) and short insertions/deletions (indels). Two variant callers were used for germline variant calling; the GATK HaplotypeCaller [25] and VarDict [26]. Reported variants were annotated with Annovar [27] and Oncotator [28] annotation programs. Tumor specific variants were assessed by somatic (paired; tumor vs. control) variant calling. For this purpose, we used GATK MuTect2 (SNVs), Scalpel [29] (Indels), and VarDict (SNVs and Indels) variant callers. The annotation of somatic variants was performed with the addition of the COSMIC database [30]. Overview of the bioinformatic pipeline is depicted in Figure 3.

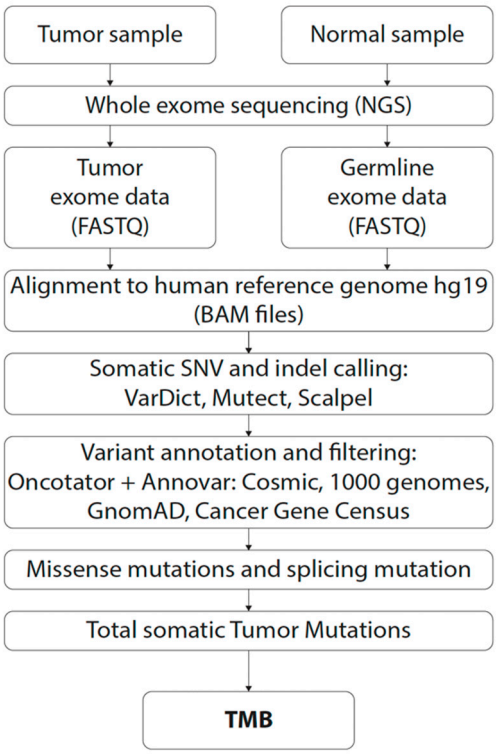

Figure 3. Workflow for tumor mutational burden (TMB) assessment by WES in this study.

4.5. Tumor Mutational Burden Estimation

An annotated list of somatic variants from the previous step was used to assess the TMB. We chose to compare two methods of TMB estimation, both based on publicly available approaches.

Method 1 (M1)—In our laboratory, we only consider somatic single nucleotide variants (SNVs) for TMB calculation from WES data, since indels (short insertions and deletions) tend to be called with high false positive rates and could potentially skew the outcome. Additionally, two bases before and

after each exon are considered as splicing mutations. Synonymous variants are filtered out, as they do not fit the definition of TMB. Finally, variants with variant allele frequency (VAF) of less than 5% are also filtered out. This approach is also used by MSK-IMPACT NGS panel.

Method 2 (M2)—This approach, used by the Foundation Medicine Inc. (FMI) targeted panels (e.g., F1CDx [2] as well as F1Heme), defines TMB as the number of SNVs (including synonymous variants) and indels in the coding regions of targeted genes. However, splicing variants are not included. A 5% cut-off for the VAF was also applied.

For the final TMB calculation, in both methods, the sum of variants remaining after application of the all filters, is then divided by the size (in megabases) of the target region from which the variants have been assessed. The target regions together with their sizes are listed below.

Both methods were applied to the three target regions (as shown in Table 5):

1. All coding sequences (whole exome; 35 Mb; using M1 for TMB calculation);
2. The coding sequences of genes analyzed by the FMI (F1CDx panel; 324 cancer-related genes; 0,8 Mbl using M2 for TMB calculation);
3. The coding sequences of genes analyzed by the Memorial Sloan Kettering Cancer Center (MSK-IMPACT; 468 cancer-related genes; 1.22 Mb; using M1 for TMB calculation)

The coding region locations on the hg19 genome were downloaded from the UCSC web site.

4.6. Comparative Study with the Foundation Medicine Inc. (FMI) Sequencing Service

FFPE tumor tissue samples of 34 patients who were previously examined by WES in our laboratory and were sent to the FMI for the FoundationOne Heme (F1Heme) test, which is recommended by vendor for pediatric tumors. In the nine cases, WES was performed using fresh frozen tissue, while different FFPE samples were sent for the F1Heme test. These specimens are indicated in the summarizing tables (Table 3) with the TMB results.

5. Conclusions

We present a study, where, for the first time in the context of pediatric tumors, the reliability of TMB estimation across multiple pediatric cancer types using real-life WES and in silico analysis of two major targeted gene panels was assessed. We confirmed a significant technological variability introduced by different laboratory technologies and various settings of bioinformatic pipelines. These results may provide valuable information for improving the accuracy of TMB estimation based on targeted gene panel sequencing in a diagnostic setting. Our study confirmed previous observations from adult tumors and thus supports the incentive to establish concordance between assay platforms used across different clinical trials in order to achieve a successful real-world implementation of TMB testing. To this end, worldwide efforts to ensure the harmonization of TMB assessment are ongoing [31–33].

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Review

Future paradigms for precision oncology

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ABSTRACT

Research has exposed cancer to be a heterogeneous disease with a high degree of inter-tumoral and intra-tumoral variability. Individual tumors have unique profiles, and these molecular signatures make the use of traditional histology-based treatments problematic. The conventional diagnostic categories, while necessary for care, thwart the use of molecular information for treatment as molecular characteristics cross tissue types.

This is compounded by the struggle to keep abreast the scientific advances made in all fields of science, and by the enormous challenge to organize, cross-reference,

and apply molecular data for patient benefit. In order to supplement the site-specific, histology-driven diagnosis with genomic, proteomic and metabolomics information, a paradigm shift in diagnosis and treatment of patients is required.

While most physicians are open and keen to use the emerging data for therapy, even those versed in molecular therapeutics are overwhelmed with the amount of available data. It is not surprising that even though The Human Genome Project was completed thirteen years ago, our patients have not benefited from the information. Physicians cannot, and should not be asked to process the gigabytes of genomic and proteomic information on their own in order to provide patients with safe therapies. The following consensus summary identifies the needed for practice changes, proposes potential solutions to the present crisis of informational overload, suggests ways of providing physicians with the tools necessary for interpreting patient specific molecular profiles, and facilitates the implementation of quantitative precision medicine. It also provides two case studies where this approach has been used.

INTRODUCTION

The conventional approaches to cancer therapy have been until very recently based on eradicating cancer cells by three modalities - surgery, radiation and chemotherapy. While this approach improved outcomes for children with acute lymphoblastic leukemia where survival rose from 20% in the 1950's to about 95% now, it was much less effective in solid tumors and adult leukemias. In these more genetically complex cancers, some modest initial improvements in survival rates were achieved, but even those modest gains have been stagnating since the late 90's. Many different reasons contribute to the treatment resistance of solid tumors and adult leukemias, but chiefly among those are: 1. the genomic complexity and heterogeneity of these entities, and 2. the protective effect of the host / tumor microenvironment.[1, 2] Novel, molecularly-based treatment modalities target not only tumor cells, but also the tumor cell-induced changes in the tumor microenvironment. In addition to those agents directed against tumor cell epitopes and receptor tyrosine kinases, there are monoclonal antibodies directed against endothelial growth factors and receptors, inflammatory cells and immune surveillance cells. All of those can be combined to correct the tumor/ microenvironment interaction, and not only sensitize to existing therapies but to effectively target the developmental end-stage characteristics of tumorigenesis.

The term biologic agent is therefore quite broad. It should be considered synonymous with "biological response modifiers", "targeted agents" or "molecularly-guided therapies", as well as with other terms used in the broader scientific literature to describe agents that target an otherwise physiological biological events "hijacked" by the tumor for growth benefit. The physiological mechanisms used by tumor cells for survival, i.e. inflammation, angiogenesis, immune system and regenerative pathways, have not been considered as targets in the past, even though wide-ranging spectrum of agents exists for their modulation. They include inhibitors of growth factor pathways, angiogenesis inhibitors, enhancers of pro-apoptotic signals, immune response modifiers, adhesion inhibitors, proteasome inhibitors, signal transduction inhibitors and any other agents targeting a defined biological process in the cancer tissues.

Unfortunately, while all these new insights have come to the forefront of cancer science, their implementation to clinical practice has been quite slow. The understanding that cancer-specific biology may be less dependent on the tissue of origin, and more dependent on a genomic (molecular) signatures, represents a paradigm shift in thinking. This new definition accepts cancer not as foreign tissue, but rather as a natural consequence of lifelong accumulation of molecular alterations, lending credence to therapeutic approach that considers cancer a chronic disease. Unlike the present goal of cancer eradication in a manner similar to antibacterial therapy; scientists now accept that cancer may be managed as a lingering chronic illness influenced by the inflammatory, immune and angiogenesis phenotype of the host. Scientists continue to identify the many molecular lesions that can lead to cancer progression and recognize that each tumor harbors its own genomic signature.[3] The basic question that remains to be answered is which part(s) of the molecular signature are related to the primary oncogenic event, and which are secondary.

The traditional picture of a linear evolution of a cancer through clonal expansion driven by accumulation of sequential mutations inherent to the cancer clone has now been nuanced by the influence of tumor microenvironment. Most cancers are a mixture of cancer cells and normal host cells that have been recruited to the site, or that have been induced to action by oncogenic changes occurring in cancer cells during malignant

transformation. In genetically complex forms of cancers, it is difficult to define a specific "driver gene" within the multiplicity of gene alterations, unless one can evaluate the quorum of signals within the tumor microenvironment. A vastly improved ability to establish the hierarchy of genomic alterations present in the tumors of individual patients will be needed for a correct analysis and interpretation of biological information.

Despite the incomplete and continuously amended molecular information, and notwithstanding the fragmented understanding of its usefulness for effective anti-cancer therapies, many molecularly-based therapies have been implemented with spectacular success. Yet, as the example of imatinib demonstrates, the deployment of targeted therapy - from its discovery to standard of practice clinical use - can take more than thirty years in the present clinical climate.[4] Even in the case of CML, a cancer with a single therapeutic target, the traditional route to clinical implementation of *bcr/abl* complex inhibitors was uncomfortably slow. The process may be streamlined in rare diseases - the use of denosumab (inhibitor of RANKL) for the treatment of giant cell tumor of the bone - but the implementation of even a single agent therapy is filled with trepidations and insurance denials. It is therefore not surprising that for those diseases with activation of more than one molecular pathway, the implementation of molecularly-guided therapy remains challenging.

Therapeutic strategies incorporating inhibition of multiple molecular pathways will need to address the considerable differences in tumors between individuals, the heterogeneity within a single tumor, as well as the differences between the primary tumor and its metastatic lesions. Numerous and quite comprehensive catalogues of somatic mutations obtained by comparing a patient's tumor DNA/RNA sequences to his/her germline DNA/RNA[5, 6] indicate a great deal of heterogeneity in cancer genome evolution across different tumor types, across individual patients with the same tumor type, and even within a tumor.[7, 8] Considering this heterogeneity, the present appeal of enhancing the traditional site- and histology-specific treatment protocols with a more personalized approach (ie. precision medicine), can be more easily understood.

Scientists[9, 10] and leading politicians[11] have recognized that supporting progress toward precision medicine and increasing the use of biological therapies holds a strong promise of not only improving health outcomes,[12] but also of potentially improving cost effectiveness of cancer therapies.[13] The concept of precision medicine, as heretical as it may have initially sounded in cancer therapy, is not foreign in medicine. We test for antibiotic sensitivity, and we match blood for HLA subtypes in transfusion and transplantation medicine, and it is not surprising that our cancer patients are beginning to demand the same.[14] Ultimately, effective, precise, target-tailored medicines may abolish the use of old-fashioned cytotoxic treatments, or at least eliminate the need for maximum tolerated doses of radiation and chemotherapy. The implementation of these new treatment modalities will, require a number of necessary changes to the oncological practice and research in oncology. We will need to:

1. change clinical trial design in order to obtain efficacy data from *n* - 1 trials
2. provide and interpret large data while maintaining excellent data integrity
3. develop novel mathematical approaches for establishing hierarchy of genomic alterations in individual tumor samples
4. provide combination therapies based on pathway analyses
5. avoid combinations with maximum tolerated doses of chemotherapy: the argument for low dose (metronomic) chemotherapy backbone

THE NEED TO CHANGE CLINICAL TRIAL DESIGN IN ORDER TO OBTAIN EFFICACY DATA FROM N - 1 TRIALS

Medical practice is a conservative vocation, and one of the most often repeated quotation in medical lore is: *Primum non nocere* ("first do no harm"). As such, in order to facilitate the translation of precision medicine to practice, sufficient evidence about precision medicine being as good or better than present therapies is requisite for the larger scientific and medical community to use the therapy. Unfortunately, over the last 40 years various regulations, were instituted in order to protect the public from unfounded claims of cure. While these were initially created for the benefit of the patient, they have led to a very inflexible structure of clinical trials - one that is no longer optimal for testing of new biological agents. Present clinical trials involve the addition of a single new agent to standard, established, maximum tolerated dose of therapy. To arrive at such a trial, the new agent must first go through a dose finding (dose escalating) trial (Phase I), which determines its maximum tolerated dose (MTD). The need to know the MTD is based on the well ingrained notion that the relationship between dose and cancer cell kill is linear[15] and more must be better. The notion, even though disavowed by the same scientist that first introduced it[16, 17], continues to be very dominant in oncology, even though some oncologists have begun using lower doses of chemotherapy in combination with targeted therapies.[18-21]

Once the MTD is defined in Phase I trial, the agent is put through an early efficacy trial (Phase II), before proceeding to a randomized, double blind, placebo-controlled (Phase III) trial to validate its efficacy, and to post-marketing surveillance studies (Phase IV). While Phase I-IV trials were informative for evaluation of the

conventional surgery/chemotherapy/radiation approach, it is not optimal for biological agents where optimal dose is not the MTD and where toxicities are minimal.[22] This particular point is further discussed in section 5.1, and represented graphically in the Figure 1. Phase I-IV clinical trial design may not only be unsuitable for testing biological agents, they may be detrimental to the testing of biologically based therapies because most biologic agents sensitize to chemotherapy and radiation, and thus heighten the toxicity in the combination arms.[23, 24]

A body of pre-clinical and clinical evidence indeed suggests that the relationship between the dose of a biologic agent and its effect is NOT linear.[25, 26] It is most commonly U-shaped. One of the earliest publications suggesting this phenomenon showed that the effect of interferon alpha 2B differed at low, medium and high doses[27] (see Figure 1A). This was subsequently found to be true for most biologic agents, especially those that depend on receptor/ligand interaction. Once all receptors are engaged, and the full effect achieved, any further increase in dose leads to off-target effects rather than further receptor inhibition. The excess of drug therefore intensifies toxicities. For example, while the effect of TGF beta1 at low doses is anti-tumorigenic, its effect at higher levels is pro-tumorigenic, creating a U-shaped response curve (see Figure 1B).[28] This characteristic u-shaped response curve of biological agents, termed hormesis,[26] further illustrates that levels and function of biological agents influence the equipoise of several pathways, and can be tumor suppressive or tumor promoting.

The doses of biological agents should therefore be determined by the optimal biologically effective

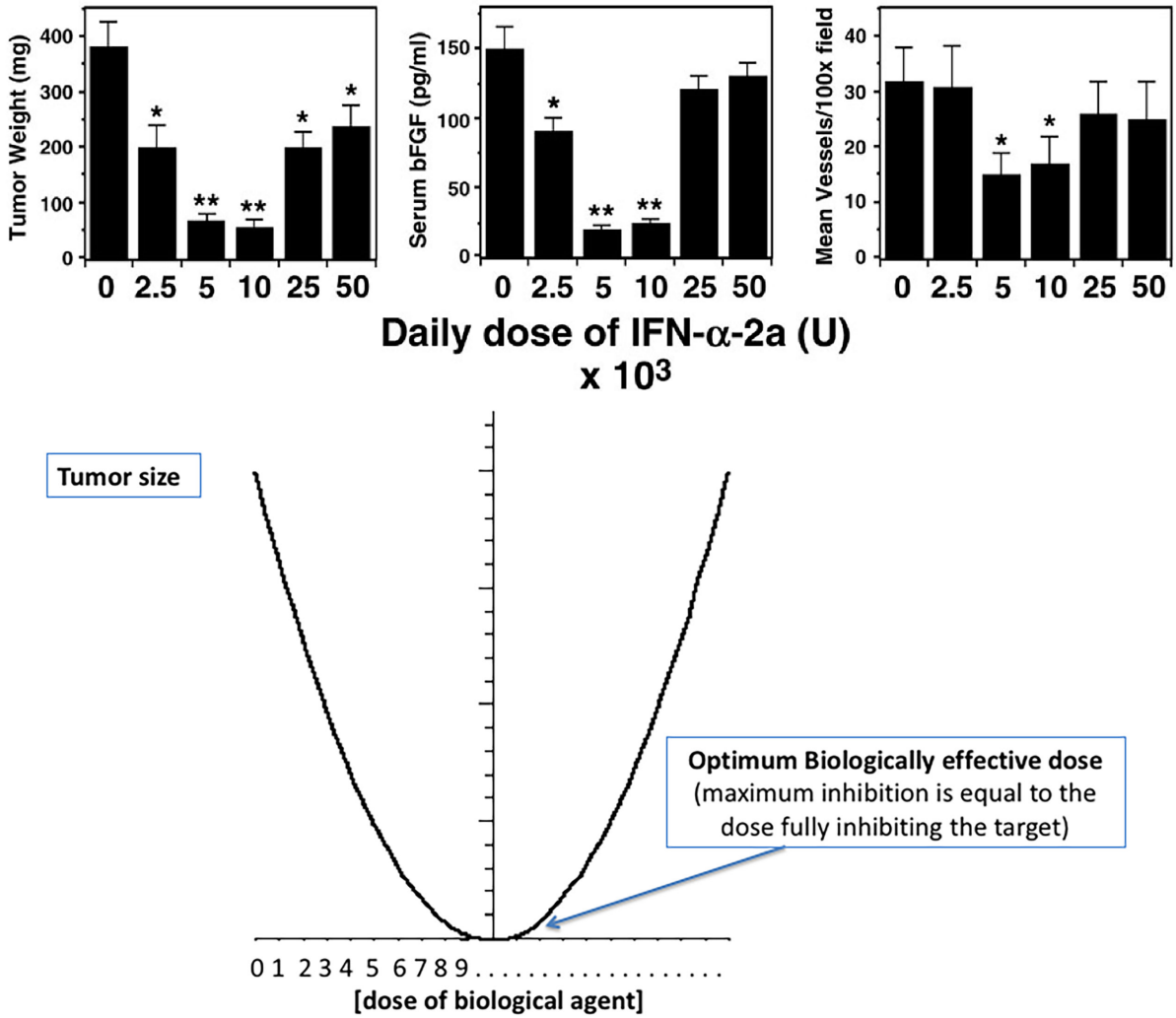

Figure 1: The U-shaped curve associate with the effect of biological therapies. Unlike the linear relationship between dose and cell kill assumed in the early work of Skipper and Schabel[15] - the effect of a biologic agent may differ at low and high doses. Panel A is an adaptation of figure first published by Slaton[23] in 1999. The optimum biologically effective dose is often a medium rather than maximum dose. This U-SHAPED CURVE may facilitate the initial up and the subsequent down-regulation during physiological biological processes. In a stress response a linear increase of interleukins is desired during the initial stress, but a relaxation needs to follow in presence of excess ligand.

dose, rather than by a maximum tolerated dose, and the Phase I/II trials are not suitable for the introduction of a biological agent to clinic. In the case of biologic agents more is not necessarily better, and dose escalations using the traditional Phase I trial may not only be inappropriate, they can be detrimental, because the effect of the biological agent at high doses may be opposite to the desired effect.[25, 26] The change in pharmacodynamics of metronomically dosed vinblastine vs MTD vinblastine provides a very good example. The dose of vinblastine used for inhibition of angiogenesis is many folds lower than the anti-proliferative dose of vinblastine (~6mg/m^2).[29]

The fact that Phase I trials are in general meant to establish dose-limiting toxicities rather than offer therapy is something most patients may not be able to appreciate when a Phase I trial is presented to them as the "last option". The chance of cure or even of a positive response is very small, especially *in situ*ations where the intended target is not tested for and may not even be present. While some early efficacy trials of targeted agents for relapsed cancers may show some effectiveness,[30] the response is rarely sustained.

The role of a randomized, double-blind placebo controlled trial (RCT) is similarly questionable in an era where precision medicine is available. An RCT is in principle a comparison of two populations, one with and the other without the tested agent. Its goal is to find an agent that would be effective for the largest percentage of the general population, rather than optimize therapy for an individual. Because identifying the best treatment for an individual is so fundamentally different from a treatment that performs best at the population level, it is highly unlikely that Phase III approaches will be able to capture the outcomes of targeted therapies in precision medicine.

There is an early level of recognition of the need to revise the present model of clinical trials. Timely changes to clinical practice have been suggested by the recent National Cancer Institute Precision Medicine Initiatives for the new National Clinical Trials Network,[31] but most molecular testing continues to be used only as means to streamline the enrollment in clinical trials. In order to accommodate the $n = 1$ trial model, early discussions have been initiated about creation of a "cancer knowledge network",[10] where information from the numerous case studies of truly individualized cancer treatments could be shared and evaluated. A case in point is the early effort to collect data from patients using targeted therapies in the NCI-Molecular Analysis for Therapy Choice (NCI-MATCH) Trial. In this trial, which opened in August 2015, analyzes patients' tumors to determine whether they contain genetic abnormalities for which a targeted drug exists (that is, "actionable mutations") and assigns the patient to a clinical trial based on one of the detected abnormalities. While the trial will make some data available, its limitation lies in its traditional trial design. The trial suffers from two shortcomings; one, it is likely that of the hundreds of patients tested, only very few will find a matching clinical trial, and two, even though the tumor tissues will be analyzed for more than 4,000 different variants across 143 genes, patients with more than one genomic abnormality will still be enrolled on a single agent therapy trial, ignoring the actual tumor biology. This approach does not change the paradigm, as it does not address the complexity of tumor biology, heterogeneity and especially not the need for pathway analysis in cancer therapy.

A special problem in clinical studies is the current practice to include at first instance only relapsed and refractory patients. As mentioned, malignant cell proliferation is under control of the primary oncogenic event, but secondary (acquired) changes may independently control further malignant cell proliferation. The chance that analysis of tumors in newly diagnosed patients may elucidate the basic oncogenic driver(s) and the respective pathway(s) is much more likely. In this respect, newly diagnosed patients with cancers where the prognosis is poor should be considered for individualized therapies before resorting to the present standards. In children with poor prognosis disease, a well designed up-front window therapy, would clarify response to biological agent(s) more clearly. Examples where these studies should be considered are children with metastatic sarcoma, brain tumors or neuroblastoma where up 80% of children die despite elaborate standard chemotherapy and radiation protocols. To identify the basic oncogenic driver(s), all newly diagnosed malignancies would need additional molecular analysis as mentioned below.

A POTENTIAL SOLUTION

To remedy the difficulty of collecting individual case study data we propose formation of consortium(s) of pediatric and adult institutions providing a standardized approach to selection of targets aided by computer assisted information processing and facilitated through an online tumor board review. The outcomes of the individual cases within the consortium(s) can then be pooled, evaluated, and used to inform selection of targets for future patients in real time (Figure 2). It is unlikely that all collaborative groups will be able to use the same tissue biomarker analysis outside a collaborative clinical trial. Only a collaborative, synchronized evaluation can lead to the meticulous collection and sharing of the DNA/RNA/Protein tissue analysis, that can lead to standardized selection of targets and therapeutic agent combinations, and where meticulous collection of the respective outcomes can be done.

The approach of this consortium has some similarities to the efforts extended by the ECOG-ACRIN

Cancer Research Group, NMTRC, SWOG, Alliance for Clinical Trials in Oncology, NRG Oncology Group and the multiple sites participating in the NCI National Clinical Trial Network for establishing the MATCH trial. But it differs, in its use of using bio-marker driven, molecularly-targeted metronomic combination therapy. The consortium(s) stresses the use of a multi-target, multi-modality approach rather than enrollment on single agent trials. The hope is that sufficient amount of data will be accumulated to provide the necessary evidence to inspire other organizations to extend the examination of tumor tissue to include genomic, proteomic and metabolomics examination of the host as well as of the tumor, and promote individualized cancer therapies. Because only a very small number of patients is going to have overlapping molecular alterations and as such require the same combination of agents, traditional population-based statistical approaches comparing two disparately treated groups may not be applicable, and novel statistical approaches using predictive models of cancer growth are going to be needed. The data from all individual patients treated by a precision medicine approach will be stored in a single de-identified database to be shared not only with the consortium members but also with other clinicians and researchers interested in using targeted approaches.

The additional benefit of sharing information of these N = 1 trials is going to be learning about the changed pharmacokinetics as combinations of different agents are being used. Pharmacokinetic studies are an integral part of present PhaseI/IV clinical trial structure. If we remove this resource, alternative experimental procedures that would allow for establishing clearance and biodistribution of these biologic agents will be needed. We will need to provide the clinicians with means to be able to quickly identify the key factors that govern absorption, distribution, metabolism, and excretion of the individual biologics, [32] the pharmacogenomics, [33], as well as the effect of using combinations of agents. Consideration will need to be given to developing new intelligence-enabled tools for quick dose adjustments if more than one cyp3a4 or other members of the cytochrome P450 family involved in drug metabolism, are being used in the therapeutic regimen.

The information collected would, in addition to traditional outcome measures such as survival, response, and toxicities, include information about quality of life and health care costs. The outcome database could thus be used to not only inform future selection of therapeutic agents and their combinations based on response, survival and toxicities, but also aid in formulating fiscally responsible clinical strategies based on cost-effectiveness models.[13]

THE NEED TO PROVIDE AND INTERPRET LARGE AMOUNTS OF DATA WHILE MAINTAINING EXCELLENT DATA INTEGRITY

However brilliant the physician may be, there is no way he/she is going to remember the millions of possible genetic variants and what each of those variants may mean for the individual patient. Moreover, given our continuously evolving understanding of the genomics, proteomics, metabolomics and other characteristics of tumor growth, it is unrealistic to expect any individual to remain current and on top of new discoveries. Invariably, in order for physicians to access and make use of the vast and constantly emerging information, she/he will need to use a variety of computational tools, and have access to a well-maintained computational support infrastructure. While initially, the focus of this computational infrastructure may be on tumor genomic signatures, and on genomic backgrounds of the hosts, it should eventually incorporate for a true personalized medicine application all of the patient's medical history, family history, dietary history, and exercise/activity information.

To implement precision medicine – and incorporate individual differences in genomic make-up and individual biological characteristics into treatment decisions – we will require the development and easy access to large-scale genomic, proteomic, biologic and health information databases. While some protein-protein interaction (PPI) networks are already publicly available on the Internet,

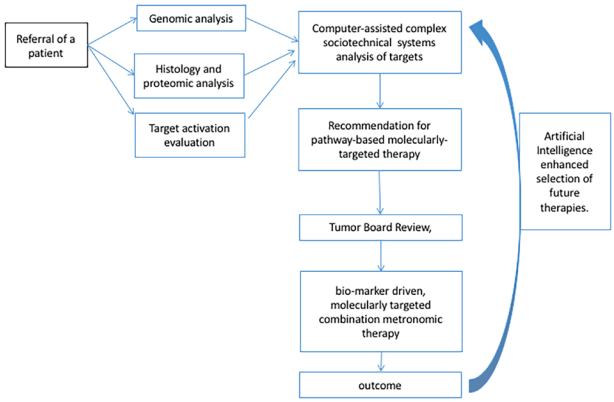

Figure 2: Pathway to combination targeted therapy design. The ability to evaluate outcomes of combination targeted therapies is dependent on the ability to standardize selection of therapeutic targets and low-dose metronomic backbones. The diagnosis of patient's molecular profile should be based not only on the genomic analysis of the patient's and the patient, but also on detecting the target proteins and their activation in the tissues. In order to incorporate, and consolidate the vast amount of information computer-assisted complex sociotechnical systems need to be employed to provide tumor boards with up-to-date information about the best molecular targets. Finally, to continuously improve the quality of the information provided to tumor boards, AI should be used to inform future decisions.

they are, at least at present, mostly complex interaction maps developed by academic biologists over the last 50 years. Of concern is that because they are maintained by academic institutions with varied levels of funding, they may be of varied levels of information integrity, and of different ability to integrate emerging information or to provide for any corrections/additions driven by new information. Due to the clear and potentially immediate impact precision medicine can exert on cancer therapies much of the information in these databases are dedicated to oncology. However, the long term goal should be to generate a broad ranging source of information about diseased and physiologic states that would be useable for general medical purposes.

A POTENTIAL SOLUTION

To address the difficulty accessing, curating and interpreting large data, a clinician-relevant computer assisted search of available information of the publicly available databases needs to be created. While more information than ever is available to the clinician, the information is not only overwhelming, it is also dispersed across varied and copious sources, few of which are geared to clinical applications. Automated systems that can trawl, collect and align available relevant information and provide assistive interpretations for clinicians would significantly alleviate this problem. We can begin by accessing available information in publicly available academically or National Institute of Health curated databases and incorporate cancer knowledge networks as they become available. Such augmented human intelligence can improve the ability of an institutional tumor board to understand and interpret all of the available gamut of molecular information and remaining current on published medical information.

The computerized system, containing a variety of artificial intelligence technologies can integrate a wide variety of information and apply an "understanding" of cancer biology in order to guide a tumor board in designing the most effective therapy for each of its unique patients. The system can do so by incorporating and cross-referencing information from multiple modalities, integrating this information in a clinical oncology context, and providing mathematical analysis of molecular pathways relevant to the patient's specific (identified) molecular changes. The information incorporated into this stream can come not only from traditional academically curated databases, but also from medical and popular scientific literature sources, public media as well as health/fitness tracking databases as recovered through social media. The information relevant to the individual patient can therefore superimposed onto a consolidated and highly cross-referenced informational stream providing the safest avenue for using the most up-to-date and continuously extended by emerging information.

THE NEED TO DEVELOP NOVEL MATHEMATICAL APPROACHES FOR ESTABLISHING HIERARCHY OF GENOMIC ALTERATIONS IN INDIVIDUAL TUMOR SAMPLES

While the advent of genomic testing - whether by a panel of genes or the entire genome - offers tremendous potential in clinical decision-making. There is presently a dearth of choices in ways to interpret and apply the information to the clinic. Scientists and clinicians are besieged with methods for differentiating between *driver genes* and *passenger genes*, realizing that not all gene alterations detected in cancer tissues are of equal importance. The conservative approach has been to use an expert-approved panel of candidate oncogenes and tumor suppressor genes in clinical testing. However, most candidate gene panels test only for gene alterations well documented in the literature and other authoritative sources. Those targets are 'assumed' by experts to be necessary for cancer progression based on the fact that some of these candidate genes have been around for decades. They may be considered universal driver genes just by virtue of our familiarity with them and their commonness. While these candidate approaches help alleviate the information glut, they are based on insufficient information given our relative paucity and incomplete knowledge about the role genetic mutations may play in the host, in tumor specific host tissues, and/or in cancer biology. While $BRAF^{V600E}$ and $BRAF^{V600K}$ mutations are established driver genes for neuroectodermal tumors such as melanoma, the use of BRAF fusions, and non $BRAF^{V600E}$ or non $BRAF^{V600K}$ gene alterations in gliomas will have to be established.[24, 34]

To use and organize the continuously emerging and heterogeneous information being deposited into genomic (The Cancer Genome Atlas, TCGA; Gene Expression Omnibus, GEO; the NCI's Database of Genomic Structural Variation; dbVar etc), proteomic (UniProt, Swiss-Prot end may others), and metabolomics (Kyoto Encyclopedia of Genes and Genomes, KEGG; and other) databases, as well as the concerted effort to identify and catalog genomic vulnerabilities across hundreds of cancer cell lines (Broad Institute's Project Achilles), new computational tools for repeated and potentially automated analysis of large data sets need to be developed.

A POTENTIAL SOLUTION

The impetus lies in improving the ability to select the most appropriate therapeutic target(s) for a particular patient. This necessitates development of novel approaches for large genomic or proteomic data analysis through multidisciplinary collaborations between mathematicians, physicists, statisticians, pharmacists, physicians,

bioinformaticians, artificial intelligence developers, biologists and software developers. The trans disciplinary process is mandatory in order to cover the end-to-end process, from cancer diagnosis, to testing for genomic alterations, to selecting appropriate targets, to analyzing pathways involved in cancer progression, to the design and administration of therapies. The motivation should be improving the ability to select the most appropriate therapeutic target(s) for a particular patient.

There are two approaches to this. The first is more established and uses high-throughput statistical analysis (bioinformatics) of genomic data such as mRNA transcriptomes or RNA Seq from tumors of a population of patients with the same disease.[35-39] This approach provides the means to identify the most frequent genetic alterations in a population. The alternative approach applies novel mathematical and physical methods to determine how the individual patient compares to the genomic information derived from the population studies.[40, 41] While it is expected that both approaches will merge in the not too distant future, they remain distinct at present and exist in two separate solitudes. Yet, in order to base a treatment decision on the unique molecular signature of the patient's tumor, an a priori resolution of the detected molecular alterations using both methods is an absolute starting point for the process.

One previously described novel physical method for prioritization of targets applies a thermodynamic interpretation to gene expression, and then uses a topological filter to identify a set of potential therapeutic targets by their predicted effect on survival.[42, 43] The method makes use of publically available protein-protein interaction networks (PPI networks). These PPIs are online repositories of interaction datasets compiled by international teams of academicians and researchers, and comprehensively curated into networks akin to telecommunication or social network maps. The thermodynamic entropy method considers these PPI networks a closed system where all interactions tend to equilibrium, and where entropy is a measure of the PPI network disorder. Because degree entropy of PPI networks for different cancers, correlates with likelihood of survival of patients with this cancer,[43] one can calculate the effect of eliminating a specific target (or eliminating multiple targets). This approach has demonstrated promising results, and points to the benefits arising from incorporating multidisciplinary perspectives to cancer models.

Another previously described method performs a pan-cancer analysis of mutated networks.[44] This unbiased and open-ended analysis had revealed 16 significantly mutated subnetworks that were not previously thought to play significant role in cancer, and demonstrated that rare combinations of mutations, across multiple PPI networks may provide new insights and new opportunities for diagnostics and therapeutics across cancer types.

The PPI approach can be used in a number of ways. For instance, one can overlay transcriptional data from a single patient onto a PPI network, or a data set from The Cancer Genome Atlas (TCGA). As an example of the later, TCGA transcription data from a population of patients with glioblastoma multiforme (GBM) was overlaid on the BioGrid PPI network. The current Biogrid Index[45, 46] version 3.3.124 (http://thebiogrid.org/), holds more than 820,000 protein interactions derived from high-throughput datasets, individual focused experiments, and from over 44,000 publications. The types of protein-protein interactions include actual chemical bonding, or temporary bonds known as secondary bonding, and the concentration of the specific proteins dictates the degree of interaction. If a protein is in limited supply, it is said to have low chemical potential, and if it is abundant, it is said to have high chemical potential. Thus, using protein concentration, we can calculate the chemical potential of each protein in the network (i.e. Gibbs free energy), compute a topological measure known as filtration threshold (an energy threshold), and "filter out" the most energetic subnetworks from the larger network and try to reduce complexity of these subnetworks by inhibiting each protein in turn. Using this strategy, the "best therapeutic targets" are those that, when inhibited, most effectively reduce the complexity of a PPI network.

As an alternative, one can superimpose patient-specific tumor mRNA transcription data (a surrogate for protein concentration) onto BioGrid, calculate Gibbs free energy for all proteins in the network, and identify those nodes with most effect on entropy. Many of these nodes may not have been identified in the specific tumor type. For example, BRACA1, an accepted therapeutic target in breast or ovarian cancer, was identified as best therapeutic target for 41 out of 342 glioblastoma multiforme (GBM) patients in TCGA,[47] even though the importance of its overexpression in GBM is unknown. Similarly SIN3 was important in 38 of the 342 GBM patients in TCGA, and SIN3 turns out to be a member of a regulatory complex in the biology of glioblastoma.[48] A total of 46 unique targets were identified using GBM transcription data from 342 patients with glioma available in TCGA.

The complex sociotechnical system[49] considered here should be designed to work with as much genetic, proteomic and biologic information as available, and involve as many fields of expertise as possible. It should be noted, that even though it is being designed for maximum efficacy in cancer (both solid tumors and leukemias/lymphomas), it can be broadened to cardiology, inflammatory bowel disease and other medical specialties as genomic information in these fields emerges. It is able to use full transcription information from the tumor tissue; subtractive transcription information of tumor tissue and patient normal tissue; proteomic analysis of the same; phosphorylation maps, methylation arrays etc. At

a minimum, it requires genetic information in the form of gene expression (transcription) microarrays or a panel of genes. Its strength lies in being able to continuously incorporate new information, as well as new mathematical and thermodynamic methods for therapeutic target prediction.

THE NEED TO PROVIDE COMBINATION THERAPIES BASED ON PATHWAY ANALYSIS

Treatment decisions are, at least in present oncology practice, made on the basis of histological diagnosis, site of tumor origin (breast, lung, prostate etc), and the familiarity of the oncologist with a therapeutic agent. Despite the documented genetic and biological differences in even histologically identical site-specific cancer types,[8] most first line therapies do not diverge from the National Comprehensive Cancer Network (NCCN) Guidelines for Treatment of Cancer and national guidelines in other countries by site. They do not incorporate RNA/DNA sequence, transcription or protein expression information. Despite the evidence that molecular signatures of seemingly diverse and distinct cancers (lung squamous, head and neck, and a subset of bladder cancers) can coalesce into a common, site-independent molecular subtype,[50] most patients are still treated according to cancer site specific protocols. If considered, new treatment modalities are used only in second or later line of therapy, when additional molecular changes may have been added to the cancer initiating event adding to the complexity of controlling cancer growth.

It is encouraging, however, that more and more oncologists are looking for safe and rational ways to incorporate genomic and biological information into first line therapies and individualize treatment protocols. This is especially true for oncologists treating patients with poor prognoses cancers such as sarcomas or brain tumors. But the approaches differ widely. The phrase "precision medicine" or "targeted therapies" are employed to describe a wide range of approaches in clinical oncology such as:

1. Targeted therapies used because a specific, single molecule is presumed to be present on the basis of previously published data (populational approach).

2. Therapies where, based on the histology of the tumor, a specific molecular target is looked for, identified and, if the mutation is present, treated as part of a single agent trial (a candidate target approach).

3. Targeted therapies that test for a panel of candidate molecules (usually an expert established panel of genes), but where a single target, selected either on the basis of its availability in a clinical trial, or on the availability of an FDA approved drug, is used (a panel of candidate targets approach).

4. Therapies that test the entire genome or transcriptome of the tumor and/or of the patient, but where a single molecular target is selected and treated.

5. Therapies that test the entire transcriptome and/or proteome and/or exome (note that the candidate approach is a subset of the full exome), a combination of molecular targets according to the 'pathway activation strategy' is selected, and all targets contributing to tumor progression are treated (the position of the authors).

It should be stressed, that using targeted agents in absence of testing for molecular alterations may be detrimental.[12] A recent comparison of outcomes of patients treated with targeted agents without testing the tumor tissues for targets (i.e. non-personalized targeted therapies) was associated with significantly poorer outcomes than even traditional cytotoxic agents approaches.[12]. The same comprehensive analysis of phase II, single-agent arms revealed that, across malignancies, a personalized strategy was an independent predictor of better outcomes and fewer toxic deaths[12] Similarly, using strategies that do not use combination therapies and thus do not inhibit the majority of molecular pathways contributing to tumor progression (the single agent approach) also provide no benefit.[51] The SHIVA prospective randomized trial[51-53] compared a personalized approach with conventional therapy in relapsed refractory adult solid tumors. This was a single-agent treatment enrolling patients on the basis of limited molecular profiling of known targetable pathways, and it was not surprising that there was no difference in progression-free survival between the molecular alteration based therapy and conventional treatment. There may be more than one reason for the reduced efficacy of a single agent approach. There is a high likelihood of missing some important targets due to limited molecular profiling, and there is a high likelihood of treatment resistance due to alternative pathways with single agent approach. The use of several molecularly targeted agents in combination with low dose chemotherapy based on comprehensive analysis of individual tumor biology is an appealing way to counteract this type of treatment resistance.

The incorporation of tumor molecular signatures information into clinical practice has not been easy, and for most physicians the most acceptable manner of using tumor molecular signature information is to screen for commonly occurring alterations and to enroll the patient on a clinical trial using the particular inhibitor. While this may be a practical and rational solution, the approach is inadequate for patients with complex genomic signatures consisting of more than one gene alteration. With the exception of chronic myeloid leukemia (CML), gastrointestinal stromal tumor (GIST), dermatofibrosarcoma protruberans (DFSP), or other similarly rare cancers, single mutations rarely account for the complexity of cancer biology, or for the secondary gene activation(s) caused by alterations within the tumor microenvironment. The protection of cells from xenobiotic such as cytotoxic agents do not

require a mutation, commonly an increased expression (or activation) of molecular pathways already encoded in the genome is sufficient for emergence of resistant clone. As such targeting a single gene alterations is unlikely to be effective in most tumors. As one pathway is inhibited, an alternate pathway is activated or additional genomic alterations are acquired.

A good example is provided in targeted treatment of melanoma using monotherapy. Treatment with either vemurafenib (BRAF inhibitor) or trametinib (MEK inhibitor) alone can lead to excellent, but invariably short-lasting responses [54, 55] due to feedback activation of other pathways.[56-58] Because most oncogenic changes tend to hijack physiologic host responses such as inflammation, nullify other host defense mechanisms such as immune surveillance, and/or re-activate dormant developmental pathways for angiogenesis, immune evasion, and growth – the feedback loops are endless. Because oncogenic $BRAF^{V600E}$ can lead to melanoma cancer cell immune evasion,[59] and the reversal of this evasion by addition of PD1 or CTLA4 immunologic therapies has been shown to provide additional benefit to BRAF inhibition alone. The combination of immune checkpoint inhibitors and BRAF-targeted agents in melanoma suggests a synergistic action of these otherwise independent therapeutic modalities,[60, 61] and a much longer response duration. While there may be a specific genomic signature that corresponds to immune evasion,[62] the use of combination therapy using inhibitors of BRAF, MEK and immune checkpoint inhibitors has caused 2-year survival rates of patients with metastatic melanoma to rise to 79%.[63]

A POTENTIAL SOLUTION

A potential solution to managing the information glut and helping the oncologist to provide patients with the right combination of targeted agents and chemotherapy, is to enable them to use all of the available information. While producing complete genomic, proteomic and metabolomics datasets for each patient is not feasible at present, it has been possible in some well-funded research units to access the entire tumor and host transcriptomic information. The more complete the information provided for the analysis of the involved pathway(s), the more complete the therapeutic coverage. Unfortunately, for most physicians practicing clinical oncology today, the most feasible option is using a panel of candidate genes, because this may be covered by the patient's insurance. At lease in the US, clinical 'omic' testing is restricted to genomic panels through CLIA certified laboratories. Even though this approach carries the inherent risk that some driver genes may not be identified, and thus not included in therapy, it is a good initiating step towards the future.

The complex sociotechnical system being deployed by the authors of this manuscript maps the available molecular information from patients' tumors onto an oncology interpretation knowledge base pooled and cross-referenced from multiple sources, and weighted in PPI networks according to the unique composition of the patient's distinctive molecular signature. The combination of genetic alterations and mutational variants are matched to a series of filtered (see above) PPI subnetworks corresponding to biologic pathways relevant to cancer growth and progression, thus identifying molecular lesions that can be targeted with therapeutic intent. This complex sociotechnical system then searches the available literature and other reliable resources to find therapeutic agents targeting the identified molecular lesion(s), and minimize the number of drugs needed to inhibit all pathways within the identified PPI subnetwork. The system also considers the topology and interaction of each of the identified anomalous pathways in order to use the minimum possible drugs, and still achieve the same therapeutic result. *in situ*ations where specific genomic alterations may confer an a priori resistance to a therapeutic agent,[64, 65] the agent is eliminated.

Roughly similar to the current use of Artificial Intelligence technologies deployed in recommending movies on the basis of our previous choices, likes or dislikes, one of the AI components in this system records and documents the selection of targets, the treatment protocols and the respective outcomes in order to inform future therapeutic selections. More specifically, as oncologists and other experts on the tumor board introduce novel evidence for, or arguments against a therapeutic choice provided by the system, the information is recorded and used to refine future pathway analyses. The hope is that genomic/proteomic information will become affordable and we will include the genomic/proteomic analysis as a standard component of the electronic medical record. In turn, as more information from patient's medical record is incorporated, we will be able to consider any co-morbid conditions of the host and filter out harmful or ineffective drugs from the therapy recommendations further, resulting in improvement of the safety of our treatments.

THE NEED TO AVOID COMBINATIONS WITH MAXIMUM TOLERATED DOSES OF CHEMOTHERAPY: THE ARGUMENT FOR LOW DOSE (METRONOMIC) CHEMOTHERAPY BACKBONE

A commonly employed approach for enhancing the ability chemotherapy to fight cancer is to use chemotherapy in combination with a biological agent. An assumption is made that the inhibitory effect of the biological agent would be additive to the effect achieved by traditional chemotherapy or radiation. However, the use of biologic agents, especially those inhibiting

host responses (such as angiogenesis or inflammation), strip the anomalous cells (but also the patient's normal cells) of its defense mechanisms such as growth factors and inflammatory cytokines and lead to sensitization of all cells to DNA damaging agents such as radiation or chemotherapy. Because most mechanisms used to protect cells from xenobiota such as chemotherapy or radiation tend to activate developmental pathways already encoded in the genome, inhibition of these pathways increases toxicities whenever standard (maximum tolerated) doses of chemotherapy or radiation are used with biological agents.[66]

In a standard clinical trial, where a standard arm is compared to standard arm with the biological agent, the approach greatly disadvantages the intervention arm. The combination of the biologic agent and high dose chemotherapy, makes an already maximally toxic regimen lethal. As a result, the benefit of any tumor response will be concealed by these increased toxicities, and no overall survival benefit will be seen.[66] An example of this is the case of combining bevacizumab with standard MTD chemotherapy. While the RIBBON2 trial showed an improved progression-free survival compared to patients treated only with chemotherapy alone [PPS 7.2 months in the experimental group compared to 5.1 months in the chemotherapy only arm (p - .0072)]. The 10% improvement in overall survival rate was not statistically significant.[67] Based on this finding, the US Food and Drug Administration (FDA) revoked the approval of bevacizumab as a first line treatment for breast cancer, even though the majority of women had responded, and some remain well controlled on the drug to date.

The concept of "metronomic chemotherapy" was initially introduced in the year 2000,[29, 68, 69] and constituted a marked departure from the classic model of maximum tolerated dose (MTD) strategy. It emerged in the face of early clinical and pre-clinical evidence supporting its ability to suppress tumor growth even in cases where the cancer cell was resistant to the MTD of the used chemotherapeutic agent.[29, 68, 70, 71] Unfortunately, the concepts were poorly understood and underused. It has gained momentum however and at present it is being adopted with increasing frequency around the world,[72-74] and the website www.clinicaltrials.org now lists over 150 trials that use the word "metronomic" in their title. The mechanism of action of metronomic chemotherapy has been subject to excellent recent reviews,[75] and its value to implementation of precision medicine well documented.[22] To summarize briefly, because of the side effects induced by maximally dosed chemotherapy, the duration of the therapy has to be limited and breaks for bone marrow recovery incorporated. Furthermore, because conventional chemotherapy targets only proliferating malignant cells, a large portion of malignant cells is not affected. Only once these cells are re-engaged in the cell cycle process cytotoxic drugs are able to corrode them. Metronomic therapy implies that the use of low, continuous doses of chemotherapy in combination with biologic response modifiers not only avoid toxic side effects, but also preferentially target the host biological responses such as stromal induction,[76] angiogenesis,[68, 77, 78] immune surveillance,[75, 79, 80] and inflammation.[76] Angiogenesis and inflammation represent a physiological repair mechanisms hijacked by the proliferating tumor and actively contributing to tumor cell re-growth. The enormous success in the treatment of pediatric acute lymphoblastic leukemia, is at least partially due to the one and a half year long maintenance low dose metronomic chemotherapy.

Thus, it should be stated that in cases where up-front eradication of the cancer is not possible with MTDs, the MTD-induced up regulation of host inflammatory responses, rather than defending us from cancer, contributes to subsequent cancer progression. Because MTD chemotherapy kills only chemo-sensitive cells with each cycle, the chance of selection of a chemotherapy resistant subpopulation and recurrence is very high.

Metronomic chemotherapy, with its goal of long-term "tumor control", lower toxicity, and prevention of tumor progression (rather than immediate reduction in tumor size), may represent a more realistic strategy for cancer therapy. This is especially true for cancers not amenable to upfront cancer eradication. While slower in its onset of action (see Figure 3), metronomic dosing has demonstrated better long term tumor control, even for cancers rendered resistant to the same drug under MTD,[29, 68, 78] because the low-dose chemotherapy approach avoids selection of a resistant cancer cell population.

A very strong argument for the use of a metronomic chemotherapeutic backbone in combination with targeted therapies is the risk of metastatic growth.[81, 82] This risk of exacerbating metastases has however, only been documented with single agent therapy and only in pre-clinical murine models. It remains theoretical in clinical practice where it is usually prevented by the synergistic action of biologic agents and low dose chemotherapy. The same is true for avoiding emergence of therapeutic resistance with targeted agents alone.[57]

A POTENTIAL SOLUTION

In the coming decade(s) a background for the combination therapies will be applied for any patients with chemotherapy resistant cancer or for patients with very poor prognosis. As much information as possible should be gathered about the patient's tumor molecular signature, about the host specific germline gene alterations, and about the host phenotype as soon as possible, so as to avoid unnecessary toxicities and delays with standard therapies whenever success cannot be reasonable expectation. The hope is that data from each of these cases will be collected

and each of the individual outcomes will inform any future therapeutic decision.

CASE 1

A previously healthy 11 year old girl with neurofibromatosis type 1 was diagnosed in 2011 with a large right parietal glioblastoma multiforme following an episode of left sided weakness. She was found to have a hemorrhagic stroke, and despite a partial resection of the tumor, her hemiparesis never resolved. She was started on COG ACNS0822, randomized to Arm A, and she completed the 6 weeks of radiation and vorinostat. In November 2011 she started maintenance chemotherapy with Avastin 10mg/kg Day 1 and 14/ Temozolomide 200mg/m2 Days 1-5 for 28 day cycle. She completed 11 out of 12 cycles before coming off protocol for disease progression in October 2012.

She was started on melatonin, metformin, cyclophosphamide and erlotinib based on a proteomic analysis done at Texas Children's. She progressed again within 2 months with leptomeningeal spread to the spine, and was changed to VP-16, vincristine, crizotinib, erlotinib, vorinostat. The regimen resulted in unacceptable toxicities with myelosupression, severe mucositis, and QTc prolongation with cardiac compromise.

She was taken off any disease directed therapy in March 2013 and referred to us for molecular analysis and individualized therapy. The characteristics of the tumor at diagnosis showed activation of a number of pathways associated with cancer growth and progression. The findings and initial pathology are summarized in Figure 4. The genomic analysis revealed NF1 R1968*, BRCA1 N1355fs*10, CDK4 amplification, TP53 R175H, SOX2 amplification. Because loss of neurofibromin function leads to increase in signaling through the Ras-Raf-MAPK and mTOR pathways, [83] she was started on sirolimus 2mg daily and sorafenib to inhibit growth factors downstream from these pathways in addition to metronomic (50 mg/m2) etoposide daily. She remained stable on this regimen until December 2015 (3 years) when she had a radiological progression.

She underwent an excisional biopsy and the molecular analysis of this relapse was consistent with a radiologically, histologically and genetically more aggressive phenotype (Figure 4). In addition to the original gene alterations, she now had BRCA2 splice site 67+1G > A, ERBB3 S1074N, TSC1 splice site 364-1G > A, GLI1 amplification, STAG2 Q1167*. Her therapy was therefore changed to everolimus (Ras-Raf-MAPK and mTOR pathways), ceritinib (GLI1/sonic Hedgehog pathway), and trametinib on a metronomic chemotherapy backbone of temozolomide 25 mg/m2, and remains stable.

The case provides a good illustration about the need for multi-agent therapy based on molecular signature. It also stresses the need to consider re-biopsy with relapse as the eco-evolutionary forces within the tumor microenvironment may cause therapeutic resistance and escape from tumor dormancy.[84]

CASE 2

A 7-y old previously healthy boy with no family history of cancer was diagnosed with stage III abdominal Burkitt lymphoma in December 2014. He was initially treated standard BFM B-NHL 04 therapy, which

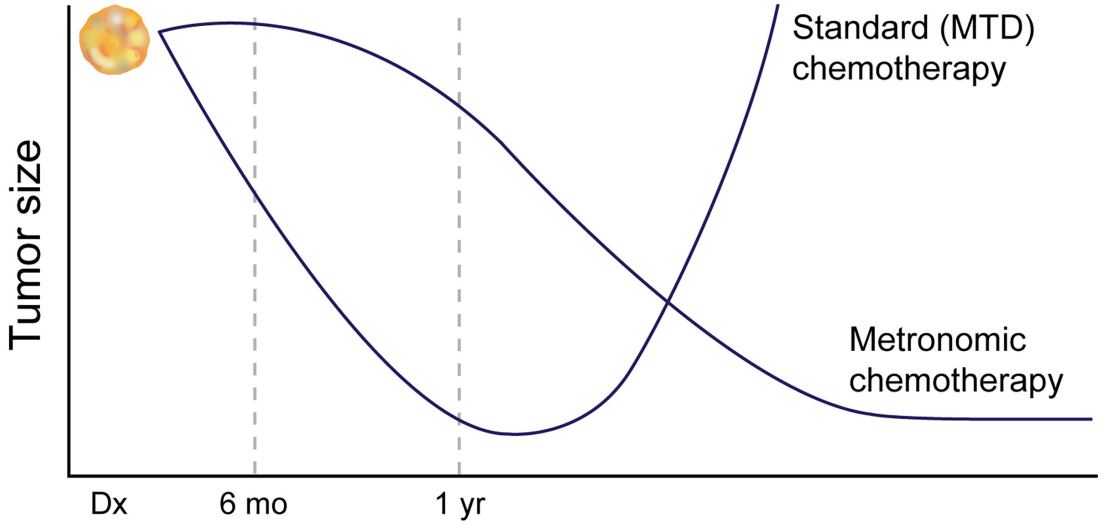

Figure 3: Comparison of Metronomic and Standard dose strategies. The onset of action of metronomic chemotherapy is slower, but because of its ability to suppress biological processes such as angiogenesis or inflammation which are often "hijacked" by the tumor for growth, and because it avoids selection of the resistant population of cells, its effects are more sustained. However if comparison of these two therapies is made before 6 months, the wrong conclusion about the effectiveness of metronomic chemotherapy may be made.

included a single initial dose of 375mg/m2 Rituximab followed with 5 cycles of BNHL 04 chemotherapy consisting of dexamethasone, methotrexate, ifosfamide, cyclophosfamide, cytarabine, etoposide, doxorubicine, vincristine as well as intrathecal therapy. After 2 cycles, he had a very good partial response reaching < 5% of the initial tumor volume. An episode of the intestinal obstruction in February 2015 led to excision, and the histology confirmed sclerosing mesenteritis, without histological or rtPCR evidence of lymphoma (the original tissue was positive for cMYC translocation). The FDG PET was borderline positive, but this was thought to be due to inflammation.

Unfortunately the child was found to have an isolated radiological progression in the same region in which the intestinal obstruction had occurred two months after completing chemotherapy. The biopsy in June 2015 confirmed relapsed Burkitt lymphoma, this time with marked areas of sclerosing mesenteritis and mesenteric panniculitis. Mutational analysis of PI3K delta subunit proved germinal mutation/variant outside the classical Activated PI3K-delta syndrome (APDS) 1 or 2 variants. The mutational activation was confirmed by testing the patient's T- lymphocytes, and the S6 (Ser235/236) phosphorylation was found to be 33 fold that of a healthy control.

While undergoing the genomic testing, the boy was started on retrieval therapy with ibrutinib, obinutuzumab and ICE chemotherapy. Unfortunately, after a transient response and disease stabilization, he had an early progression following the first cycle. Based on the finding of germline mutation in PI3K delta subunit, he got 2 weeks of idelalisib (a phosphoinositide 3-kinase inhibitor, which blocks P110δ, the delta isoform of the enzyme phosphoinositide 3-kinase). The single agent therapy led to normalization of the S6 (Ser235/236) phosphorylation in patients peripheral T lymphocytes, but he had further disease progression. It was only when the combination of high dose cytarabine/ etoposide (CyVe) with idelalisib and obinutuzumab was used that the disease was stabilized. A biopsy on 9/2015 showed a CD20 positive tumor, with high degree of proliferation and strong expression of PD-1L.

The second biopsy was analyzed using Affy GeneChip ST 1.0 and the whole transcriptome analysis confirmed increased levels of PI3K and revealed additional HR23B. Because HR23B can be used as a good predictor of response to HDAC inhibitors, valproic acid was being considered. Additional tumor specific (somatic) gene alterations in R273C and p53 were also shown.

The child, who had continued on oral ibrutinib + idelalisib and low dose cyclophosphamide since 9/2015, received palliative 21Gy local radiation. In 10/2015, based on the second biopsy findings, the nivolumab, a human IgG4 anti-PD-1 monoclonal antibody, and valproic acid, and HDAC inhibitor, were added. As of March 2016 the boy is doing very well. He has had partial response of the single residual abdominal tumor disease, and remains clinically well with Lansky score 100 and OS > 15 months. He comes to clinic biweekly for nivolumab infusions and assessments, but remains outpatient otherwise. He started his personalized therapy after his second relapse, and this 3rd EFS (7 months) is already the longest EFS, compared to 6 months post his initial standard BFM protocol and just 1 month post ibrutinib, obinutuzumab and ICE chemotherapy.

The case may illustrate a new variant of Activated PI3K-delta syndrome (APDS). At least at present the disease is not tested for and generally not recognized in children with Burkitt's lymphoma. Even if this child had a family history supporting testing for the autosomal dominant form of APDS, he would not have been found. Yet, he had an atypical germinal mutation in the gene that leads to lymphoid hyperplasia, and increases the risk of malignant transformation to B-cell lymphoma. The p110δ protein is a crucial subunit of the PI3K enzyme, and regulates activation of proliferative pathways in B-cells. As such, unless this constitutional activation can be blocked, it will be unlikely that 5 cycles of conventional chemotherapy could successfully prevent a relapse. It may be prudent, in cases where a mutational activation of an important proliferative pathway is found, to use maintenance biological therapy. This could be similar to the 2 years maintenance therapy used in childhood Acute Lymphoblastic Leukemia, which has cure rates of about 90%. It is our hope that this case illustrates a potential for keeping even children with poor prognosis due to genetically complex cancers at home. While not able to eradicate the cancer or it causative mutation, we may be able to keep them well, in school and active by prescribing a combination of low-dose metronomic chemotherapy, an immune checkpoint inhibitor, and a direct inhibitor of the activated pathway(s).

SUMMARY

Many oncologists treating recurrent, chemotherapy resistant or poor prognosis cancers have begun re-purposing anti-inflammatory agents or immune modulators. Similarly, many oncologist use direct anticancer agents in an off-label setting to target specific genomic mutations regardless of the cancer subtype. An equal number of oncologists however, due to the time required for researching the vast amount of molecular information, continue treating children with conventional therapies. But for those cancers where the present chemotherapeutic, surgical and radiation strategies fail – the option of targeted strategies should be strongly considered.

The difficulty is that physicians using targeted therapies today do so without the benefit of computational infrastructure. While we use complex sociotechnical

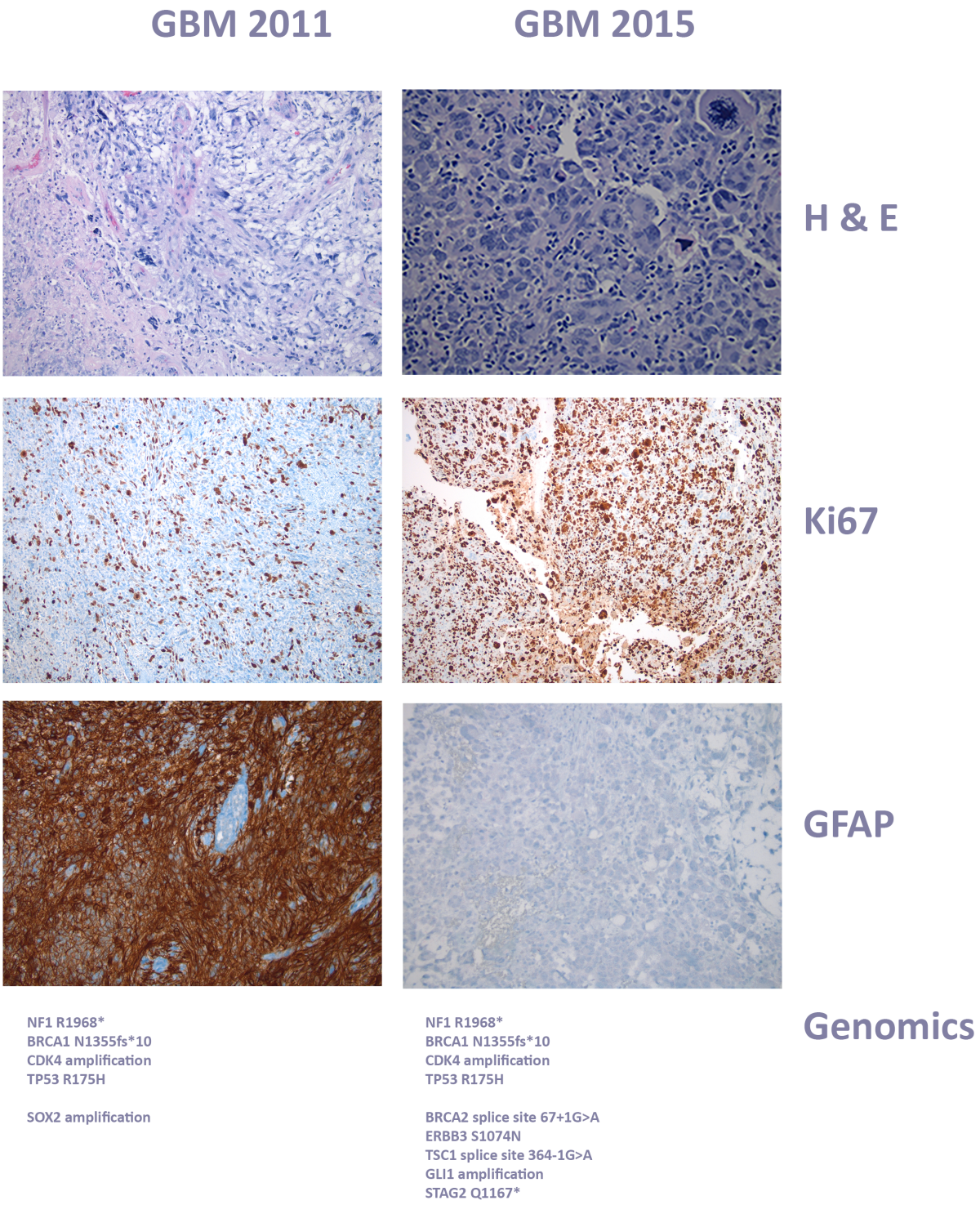

Figure 4: Histology of case 1, glioblastoma progression. The original right temporal mass resected in 2011 showed glial neoplasm with vascular proliferation, necrosis, mitosis, and numerous pleomorphic cells, including rare giant cells. At this time, there was strong and diffuse immunopositivity for GFAP, and markedly elevated Ki-67 proliferative index, consistent with Glioblastoma WHO grade IV/IV. The original lesion regressed after the initial targeted therapy with sirolimus, sorafenib and metronomic VP16, but relapsed with a new extra axial lesion. The relapsed tissue in 2015 showed glial neoplasm with numerous tumor giant cell and atypical mitoe. The tumor cells were immunonegative for NEU-N, IDH-1(R132H) and BRAFv600E, but the molecular signature had obviously evolved, adding further genomic alterations. At this time, the tumor was negative for GFAP, and the ganglional component was no longer present. Both the 2011 and 2015 specimens had shown increased lymphocytic component and myxoid background, along with tumor giant cells, but the number of giant cells was increased significantly in the 2015 specimen.

systems to manage nuclear plants and airports, we have not developed similar systems for the analysis and application of omics information. We need an efficient complex sociotechnical system that would allow us to analyze a molecular signature of the patient's cancer in minutes and select the appropriate molecular agent(s) in time for effective therapy.

We also need to abandon the present model of drug development. The present process often takes decades for each of the new therapeutic agents. Millions are spent testing each of the agents in individual Phase I-IV trials before its introduction to the clinic resulting in cost-prohibitive therapies. Most importantly however, thousands of patient lives are lost as we struggle to determine whether an agent "is clinically active" in the incorrectly designed clinical trials. A wealth of bioinformatics resources exists that can help narrow the choice of therapeutic combinations from the wide selection of already available molecular agents, and provide a treatment for a wide range of difficult to treat cancers TODAY.

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CONFLICTS OF INTEREST

There is no conflict of interest.

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Combined Biodifferentiating and Antiangiogenic Oral Metronomic Therapy is Feasible and Effective in Relapsed Solid Tumors in Children: Single-Center Pilot Study

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Key Words

Antiangiogenic · Treatment, metronomic · Trial, clinical · Child

Summary

Background: To outline an outpatient-based treatment for children with relapsed solid tumors, who already have been extensively pretreated, we defined a 4-drug protocol named COMBAT (combined oral maintenance biodifferentiating and antiangiogenic therapy). Using this protocol, we performed a pilot study to determine its feasibility in children with relapsed and/or high-risk pediatric solid tumors. **Patients and Methods:** 22 children received the COMBAT protocol. Treatment consisted of daily celecoxib administration along with daily 13-cis-retinoic acid (2 weeks on / 2 weeks off) and cycles of metronomic temozolomide (90 mg/m^2 for 42 days) and low-dose etoposide (21 days). The treatment was scheduled for a period of 1 year. **Results:** 9 of the 14 patients assessable for response demonstrated evidence of treatment benefit manifested as prolonged disease stabilization or response. The protocol medication was well tolerated with very good compliance. Only minimal side effects where observed which responded to dose modification or local therapy. **Conclusions:** The COMBAT regimen is well tolerated by patients with intensive prior therapy including myeloablative regimens. Favorable responses observed in this cohort of patients support the further exploration of this and/or similar strategies in the treatment of pediatric solid tumors.

Schlüsselwörter

Antiangiogenese · Therapie, metronomische · Studie, klinische · Kind

Zusammenfassung

Hintergrund: Mit dem Ziel der Etablierung einer ambulanten Behandlungsstrategie für intensiv vorbehandelte Kinder mit refraktären soliden Tumoren wurde ein 4 Medikamente umfassendes Therapieprotokoll, genannt COMBAT (combined oral maintenance biodifferentiating and antiangiogenic therapy), erstellt. Im Rahmen einer Pilotstudie wurde die Anwendbarkeit der Therapie bei Kindern mit soliden Tumoren, die refraktär und/oder mit einem hohen Risiko verbundenen sind, durchgeführt. **Patienten und Methoden:** 22 Kinder wurden nach dem COMBAT-Protokoll behandelt. Die Therapie bestand aus täglichen Gaben von Celecoxib und 13-cis-Retinoinsäure (2 Wochen Behandlung / 2 Wochen Pause) sowie Behandlungszyklen mit metronomischem Temozolomid (90 mg/m^2 für 42 Tage) und niedrig dosiertem Etoposid (21 Tage). Die Behandlung erfolgte über einen Zeitraum von 1 Jahr. **Ergebnisse:** 9 der 14 bezüglich des Ansprechens beurteilbaren Patienten zeigten einen Behandlungsvorteil (längere Krankheitsstabilisation oder Ansprechen). Alle Medikamente waren gut verträglich, und die Patientencompliance war gut. Die beobachteten Nebenwirkungen waren minimal und konnten durch Dosismodifikationen oder lokale Behandlung behoben werden. **Schlussfolgerungen:** Das COMBAT-Regime ist für intensiv vorbehandelte Patienten auch nach myeloablativer Therapie gut verträglich. Das in dieser Studie beobachtete positive Ansprechen unterstützt die weitere Untersuchung dieses und/oder ähnlicher Behandlungsstrategien für solide Tumoren bei Kindern.

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Introduction

The outcome of children with metastatic sarcomas, relapsed brain tumors and other high-risk refractory and/or relapsed malignancies remains poor despite the introduction of dose-intensified chemotherapy, high-dose chemotherapy and several novel therapeutic approaches, such as monoclonal antibodies and kinase inhibitors [1]. Therefore, treatment strategies which act on multiple levels including tumor cells but also the stromal milieu may be of clinical benefit [2–6]. One such strategy is the combination of cell cycle/apoptosis-inducing topomerase II inhibitors with differentiating and anti-angiogenic agents. Angiogenesis and neovascularization are required to promote and sustain malignant tumor growth [2, 3]. Angiogenic cytokines produced by tumor and stromal cells stimulate surrounding endothelial cells to proliferate locally and attract endothelial precursors from the bone marrow. Within the tumor stroma, these processes integrate towards formation of primitive (neo)vascular structures [7] that subsequently contribute to local tumor expansion and metastasis. Disruption of angiogenic signals may therefore lead to suppression of tumor growth. Inhibitors of cyclooxygenase-2 (Cox-2 inhibitors) are an example of such antiangiogenic agents [8]. In primary tumors, experimental evidence exists that cyclooxygenase-2 inhibition is a potent mechanism to reduce angiogenesis [9]. Traditional anti-angiogenic therapy is based on the inhibition of the angiogenic cascade, including angiogenic factor receptor binding, signal transduction, migration of proliferating endothelial cells and formation of vascular lumina [2, 3]. The mode of action of continuous low-dose (metronomic) chemotherapy is explained by the interference of these agents with cycling and proliferating endothelial cells in the process of tumor angiogenesis [10, 11].

Evidence supporting the effectiveness of retinoids against central nervous system (CNS) tumor cell lines [12–17] has been published. Retinoids not only inhibit cell growth but can also induce apoptosis. Observation by Olson et al. [18] showed increased apoptosis in medulloblastoma/primitive neuroectodermal tumor (PNET) cells treated with 13-cis-retinoic acid. Caspase activation and induction of apoptosis have been demonstrated in vitro in glioma cell lines treated with synthetic retinoids [19]. Preclinical data have been published showing that, in vitro, cells derived from Ewing's sarcoma with the characteristic somatic rearrangement between the genes EWS and FLI 1 can be induced to differentiate toward a neuronal phenotype by exposure to agents such as dibutyryl cyclic adenosine monophosphate (db cAMP) or retinoic acid. [20]. Similar data exist for other sarcomas [21–24].

Improved survival with acceptable toxicity was found for the maximum tolerated dose of 160 mg/m^2/day of oral 13-cis-retinoic acid (isotretinoin) given for 14 days, followed by a 14-day resting period in children with neuroblastoma treated after autologous bone marrow transplant [25, 26]. No significant responses were reported in patients with bulky disease neuroblastoma in an earlier phase II study on the use of 13-cis-retinoic acid in recurrent neuroblastoma [27]. However, in a study by Yung et al. [28], 7% partial responses, 16% minor responses and 30% stable disease were observed among 43 adults with recurrent malignant glioma treated for more than 4 weeks with 60–100 mg/m^2/day of 13-cis-retinoic acid, suggesting some degree of activity of 13-cis-retinoic acid against high-grade gliomas even in patients with bulky disease. Similar supportive data was provided in another trial [29], and combination trials combining 13-cis-retinoic acid with temozolomide in adults have been reported [30, 31].

In an attempt to set up an outpatient-based and oral treatment for children with relapsed solid tumors who already had extensive prior chemotherapy, we developed a 4-drug protocol named COMBAT (combined oral maintenance biodifferentiating and antiangiogenic therapy), containing celecoxib, temozolomide, etoposide and 13-cis-retinoic acid. The dosage was set to a low-dose range so that therapy could be maintained for a prolonged period of time. The treatment was originally intended as palliative treatment for children where no higher priority treatment was available. However, encouraged by the observed responses, we subsequently offered this treatment to children with high-risk solid tumors after completion of the standard treatment. For reporting, we set 2 disease strata: i) COMBAT as palliative treatment for children with measurable, resistant, progressive and/or relapsed disease, and ii) COMBAT as maintenance therapy for children with no measurable disease but with high-risk solid tumors, who where in first or second complete remission but had a very low probability of maintaining a long-term disease-free status because of the biological character, or the extent of the malignancy. Here, we present results of our pilot study.

Patients and Methods

Study Design and Therapy Administration

The COMBAT study was reviewed by the Institutional Review Board. The treatment consisted of 4 cycles of combined oral therapy as outlined in figure 1. Each cycle lasted for 11 weeks (77 days) so that the total treatment duration was planned for approximately 1 year. Each cycle commenced if the absolute neutrophil count (ANC) was ≥ 750/µl and the unsupported platelet count was ≥ 75,000/µl. There was a 2-week gap between cycles. Drug dosing and treatment schedule are based on previously published treatments for children with high-risk neuroblastoma [32], sarcomas and brain tumors [22, 30, 31, 33–35]. No further dose escalation has been planned for this group of patients. The safety of COMBAT administration was ensured by the following algorithm. If 2 or 3 of the first 3 patients experienced dose-limiting toxicity, the dose was then considered too high. If 1 of the first 3 patients experienced dose-limiting toxicity, then 3 additional patients were enrolled. If, after enrollment of 6 patients, only 1 experienced dose-limiting toxicity, 2 more patients were enrolled. If 2 or more patients experienced dose-limiting toxicity, then the dose would be regarded higher than the maximum tolerated dose. Dose-limiting toxicity was defined as any grade 4 non-hematological toxicity, any grade 4 hematological toxicity or any grade 3 non-hematological toxicity that did not resolve within 7 days. Toxicities were graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC),

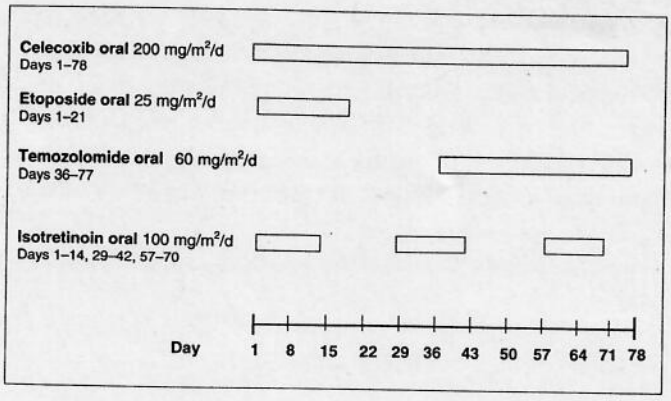

Fig. 1. Combined oral maintenance biodifferentiating and antiangiogenic therapy (COMBAT) – treatment plan.

version 2 (*http://ctep.cancer.gov/forms/CTCv20_4-30-992.pdf*), except for cheilitis which was graded according to the Common Terminology Criteria for Adverse Events (CTCAE), version 3 (*http://ctep.cancer.gov/forms/CTCAEv3.pdf*). Non-hematological dose-limiting toxicity was defined as any grade 3 or grade 4 non-hematological toxicity. Hematological dose-limiting toxicity was defined as grade 4 neutropenia lasting over 7 days, or grade 4 anemia or trombocytopenia requiring transfusion support. During COMBAT therapy, all patients had regular physical examinations and complete blood counts, and assessments of hepatic and renal function was carried out every 2 weeks. Imaging studies to evaluate the disease status were performed at the end of the 2nd and 4th therapy cycle and/or as clinically indicated. No other anticancer and/or immunomodulating therapy was used during and 4 weeks after COMBAT therapy, but patients did receive antibiotics, blood products and supportive care as clinically indicated. Antiemetics were not administered unless patients experienced nausea and/or vomiting in the course of the treatment. Patients were considered assessable for toxicity if they completed at least 1 therapy cycle (77 days).

Eligibility Criteria

Eligible patients were children who had either a relapsed or primarily treatment-resistant solid tumor or a newly diagnosed and high-risk solid tumor for which no higher priority treatment was available (table 1). For relapsed patients, only the COMBAT regimen was used. For children with newly diagnosed tumors, the COMBAT regimen was used as maintenance therapy after completion of standard adjuvant chemotherapy. Other eligibility criteria included life expectancy of more than 6 weeks, Karnofski/Lansky score > 50, adequate bone marrow function (ANC > 750/mm^3, platelets > 75,000/mm^3), adequate hepatic function (alanine aminotransferase < 2 times normal) and normal renal function (plasma creatinine and/or glomerular filtration rate). Ineligibility/exclusion criteria were as follows: history of peptic ulcer disease or significant gastrointestinal hemorrhage, history of deep venous thrombosis, hemophilia, von Willebrand's disease, other clinically significant bleeding disorder, aspirin allergy, pregnancy. An informed consent was obtained from a parent or guardian of each patient. Between November 2004 and November 2005, 22 children were recruited. Age at entry ranged from 3 years and 2 months to 19 years and 10 months with a median age of 11 years and 5 months.

Evaluation of Response

In this study, we used the Response Evaluation Criteria in Solid Tumors from the NCI [36] with the exception of CNS tumor evaluation. For this purpose, only lesions of 1 cm or larger were seen to constitute measurable disease, and a total of 5 such lesions per organ (10 lesions in total) were selected as target lesions. Measurable response was coded as follows: i) complete response (CR) – complete disappearance of disease accordi to magnetic resonance imaging (MRI)/computed tomography (CT); partial response (PR) – more than 30% reduction of the longest diamet iii) stable disease (SD) – neither CR, PR nor progressive disease (PD); PD: > 20% increase in the longest diameter of the target lesion.

For CNS tumors, CR was defined as complete clearing of radiograpl disease for at least 4 weeks. PR was defined as requiring a more than 50 decrease in the sum of the products of all two-dimensional masses fo minimum of 4 weeks. PD was defined as an increase of more that 25% the size of a measurable lesion on imaging studies, or new lesions CT/MRI in a patient who has previously attained response. SD was d fined as failure to fulfill the criteria for either CR, PR or PD. The ter very good partial remission (VGPR) was used for children with neur blastoma as defined by the International Neuroblastoma Staging Syst (INSS) [37].

Results

Toxicity

Oral medication was generally well tolerated with minim toxicity and very good compliance. No unexpected toxiciti and/or pseudotumor cerebri was observed in this study. Te mination of COMBAT therapy due to toxicity was not nece sary, and all patients recruited were assessable for toxicit Grade 4 hematological toxicity was noted in 1 child with pr gressive, metastatic neuroblastoma with massive bone ma row involvement and in another heavily pretreated child wit relapsed medulloblastoma. Non-hematological grade 3 or toxicity included only grade 3 cheilitis which occurred 7 children. The dose of temozolomide was reduced by 20% 1 child and by 40% in another due to poor tolerance report as general weakness and loss of appetite, which resolved aft dose reduction. Local therapies were sufficient in managi cheilitis in all but 1 child where the dose of 13-cis-retino acid was reduced to two thirds of the scheduled dose. Th worst drug-related toxicities across all COMBAT courses a shown in table 2.

Antitumor Activity

All 14 children with progressive diseases were assessable f response. Diagnosis, age, sex, response data and curre follow-up data, as of 1st March 2006, are summarized in t table 1. The overall response rate was 64% (9 out of 14), wi 2 CR, 2 VGPR, 2 PR and 3 SD. 35% of the patients with P (5 out of 14) received less than the prescribed 12 months therapy due to disease progression and all ultimately die 64% (9 out of 14) of assessable patients with PD and all 8 ch dren with high-risk disease continue to be progression-fre The median follow-up is 10.5 months (mean 9.8, range 5–1 for the PD group and 7 months (mean 8.25, range 6–15) f the high-risk CR group. 3 out of 4 children with neurobla toma showed objective responses with 2 VGPR. With oth solid tumors, a second CR lasting more than 11 months w achieved in an adolescent patient with relapsed thoracic m

Table 1. Patients characteristics

Diagnosis	Age at primary diagnosis, years (sex)	Previous CXT regimens, n	Previous RXT	Pattern of relapse / progression	Previous ETO/TMZ/ CRA	Previous sub/myelo-ablative therapy	TTP since primary diagnosis, months	Disease status prior to COMBAT	Best response to COMBAT	Current patient status[a]/months since start of COMBAT
PD group										
Malignant metastatic carcionoid of the liver	13 (F)	3	no	LR	ETO, TMZ	no	5	PD	SD	A/15
Osteosarcoma	10 (F)	1	no	L+M	no	yes	32	PD	PD	DoD/8
BSG	5 (M)	2	yes	L	TMZ	no	20	SD	PD	DoD/7
Neuroblastoma	4 (M)	1	yes	M	CRA	yes	21	PD	VGPR	A/14
High-grade astrocytoma	5 (M)	2	yes	L	all	no	8	PD	PR	A/13
Neuroblastoma	4 (M)	1	no	M	ETO	no	6	PD	VGPR	A/13
CNS germinoma[b]	14 (M)	0	no	–	no	no	–	–	CR	A/13
Ewing's sarcoma	9 (M)	1	no	L+-M	ETO	no	19	PD	PD	DoD/4
Metastatic/metachronous LGG	11 (M)	2	yes	L+M	ETO	no	82	PD	SD	A/10
Malignant fibrous histiocytoma	18 (M)	1	yes	L+M	ETO	no	35	PR	CR	A/11
Neuroblastoma	8 (M)	1	yes	L+M	ETO, CRA	yes	22	PD	SD	DoD/10
Neuroblastoma	18 (F)	1	no	M	yes	no	6	PD	PD	DoD/5
LGG	6 (M)	1	yes	L	no	no	13	PD	PR	A/10
Osteosarcoma	20 (F)	2	no	L+M	no	no	13	PD	SD	A/5
High-risk group										
Osteosarcoma	15 (M)	2	no	–	ETO	no	–	CR	CR	A/15
Medulloblastoma	10 (M)	1	yes	–	TMZ	yes	–	CR	CR	A/14
Intraspinal ATRT	3 (F)	3	yes	–	ETO, TMZ	yes	–	PR	CR	A/12
Medulloblastoma	17 (M)	2	yes	–	TMZ	yes	6	2nd CR	2nd CR	A/6
Medulloblastoma	12 (M)	3	yes	–	TMZ	yes	11	2nd CR	2nd CR	A/6
Medulloblastoma	15 (M)	1	yes	–	TMZ	no	–	PR	PR	A/7
Medulloblastoma	10 (M)	2	yes	–	TMZ	yes	–	CR	CR	A/13
Ewing's sarcoma	4 (F)	1	yes	–	ETO	yes	–	VGPR	VGPR	A/6

[a]As of March 2006.

[b]Patient with Smith-Lemli-Opitz syndrome; parents refused intensive standard treatment.

CXT = Chemotherapy; RXT = radiotherapy; ETO = etoposide; TMZ = temozolomide; CRA = cis-retinoic acid; TTP = time to progression; BSG = diffuse pontine brain stem glioma; LGG = low-grade glioma; ATRT = atypical teratoid/rhabdoid tumor; F = female; M = male; A = alive; L = local; LR = locoregional; L+M = local + metastatic; M = metastatic; DoD = died of disease; CR = complete remission; VGPR = very good partial remission, PR = partial response; SD = stable disease; PD = progressive disease (NCI response criteria).

Table 2. Worst drug-related toxicities reported among 22 patients evaluable for toxicity across all courses

Toxicity criteria[a]	Toxicity grade					
	None	1	2	3	4	Unknown
Hemoglobin	0	2	13	7	0	0
WBC	0	3	8	9	2	0
ANC	0	3	10	7	2	0
Platelets	0	5	7	8	2	0
Febrile neutropenia, sepsis, septic shock	20		0	2	0	0
Other infections	16	3	3	0	0	0
Neurotoxicity	16	3	3		0	0
Hypokalemia	17	4	1	0	0	0
Hyponatremia	16	2	4	0	0	0
Hyperglycemia	7	1	0	0	0	0
ALT	6	5	6	5	0	0
Bilirubin	18	3	1	0	0	0
Nausea	15	4	3	0	0	0
Vomiting	18	3	1	0	0	0
Diarrhea	20	2	0	0	0	0
Constipation	17	4	1	0	0	0
Stomatitis	15	5	2	0	0	0
Cheilitis[b]	0	2	13	7	NA	0
PTT	19	2	1	0	0	0
PT (as INR)	18	2	2	0	0	0

[a]Common Toxicity Criteria, version 2.0, NCI.
[b]Common Terminology Criteria for Adverse Events (CTCAE), version 3.0.
WBC = White blood cell count; ANC = absolute neutrophil count; ALT = alanine aminotransferase; PTT = partial thromboplastin time; PT = prothrombin time; INR = international normalized ratio; NA = not applicable.

lignant fibrous histiocytoma as well as in a child with relapsed M2 medulloblastoma lasting more than 13 months. A further 3 patients with PD showed SD for more than 5 months, with clinical improvement in all 3 patients.

Discussion

Tumor growth and progression are angiogenesis-dependent. Preclinical studies and clinical data from small series and case reports have shown that well-tolerated continuous low-dose (metronomic) chemotherapy can exert significant antiangiogenic effects per se and hence a greater antitumor activity than conventional chemotherapy with high, spaced-out bolus doses [2, 8, 10, 11]. Taking into account heterogeneity and the complexity of the angiogenesis process resulting from a dynamic balance between proangiogenic and antiangiogenic factors and the fact that different tumor cells can upregulate different inducers and/or downregulate different inhibitors, drugs given in combination rather than as single-agent treatment are of greater potential. This is supported by preclinical data [38], and by short-lasting or no responses achieved with single-agent treatment, such as etoposide [39]. The COMBAT protocol was primarily intended for patients with relapsed brain tumors and neuroblastomas where some level of evidence for single COMBAT components can be found in the literature. Evidence for the use of these components is not that strong in other malignancies, but given the fact that no other higher priority treatment was available, we decided to also offer this treatment to patients with other solid tumors in the palliative setting. We demonstrated the feasibility of administering combined oral maintenance biodifferentiating and antiangiogenic therapy in children with either relapsed and refractory or high-risk solid tumors. The to date only similar study in children was published recently by Kieran et al. [40]. This study, which used 4 orally administered drugs, 2 of which were identical with the COMBAT regimen (celecoxib and etoposide alongside thalidomide and cyclophosphamide), essentially achieved identical conclusions as we have reported. The etoposide dose in our protocol is only the half the dose used by Kieran [40]. When combining drugs for the COMBAT regimen, several factors were considered, such as possibility of oral administration, different mechanisms of action, non-overlapping toxicity and availability of preclinical and human clinical data demonstrating activity in cancer.

The COMBAT protocol proved to be well tolerated by patients with intensive prior pretreatment including myeloablative regimens. No non-hematological dose-limiting toxicity according to CTC occurred in this study. Cheilitis grade 3 according to CTCAE was the only grade 3 non-hematological toxicity recorded. Except for 1 child, local treatment was sufficient in all patients. Toxicities described here are similar to the observed toxicity profiles for drugs used in similar clinical situations as single agents or as 2-drug combination, or in similar

clinical trials [40–43]. However, doses of oral etoposide and 13-cis-retinoic acid were lower in our study compared to others, whereby the reason for the etoposide dose reduction was mainly the decreased risk of secondary malignancies [42, 43]. The overall response rate of 65% including 2 CR, 2 VGPR, 2 PR and 3 SD is encouraging and rather unexpected for extensively pretreated children. However, there only has been a short follow-up interval so far. In 3 children, the duration of response achieved with the COMBAT protocol was longer than that achieved with the previous chemotherapy using maximum tolerated doses. Responses seen in our study fulfill standard rules for phase II trials required to prove treatment efficacy and may stimulate further research focused to metronomic and differentiation therapies in children with high-risk and/or recurrent malignancies.

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Metronomic Chemotherapy with the COMBAT Regimen in Advanced Pediatric Malignancies: A Multicenter Experience

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Key Words

Children · Solid tumor · Tumor angiogenesis · Metronomic chemotherapy · Relapsed pediatric solid tumor

Abstract

Background: The outcome of children with refractory/relapsed malignancies remains poor and novel therapies are urgently required. One of the promising approaches is metronomic chemotherapy. We present the clinical results of 74 children with advanced solid tumors treated according to treatment recommendation with data registry in three European pediatric centers. ***Methods:*** COMBAT (Combined Oral Metronomic Biodifferentiating Antiangiogenic Treatment) included low-dose daily temozolomide, etoposide, celecoxib, vitamin D, fenofibrate and retinoic acid. From 2004 to 2010, 74 children were enrolled. ***Results:*** The 2-year overall survival (OS) was 43.1% (median 15.4, range 1.3–69.9 months). Of the 74 patients, 50 patients (68%) died and 24 are alive: 6 (8%) with progressive disease, 7 (9%) with stable disease/partial response and 11 (15%) in complete response. Median time to response was 6 months. Of 62 patients with initially measurable disease, 25 (40%) had radiological response or stable disease. Fourteen of 25 showing clinical benefit responded within the first 6 months. The treatment was well tolerated on an outpatient basis. Regarding non-hematological toxicity of grade ≥2, hepatotoxicity of grade 3 occurred in 8 children and grade 3 cheilitis in 16 children. ***Conclusion:*** COMBAT is a feasible and effective treatment option for patients with relapsing/refractory malignancies. The treatment is well tolerated with a low acute toxicity profile.

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Introduction

Despite substantial improvement in the treatment of pediatric malignancies during the last decades, the outcome of children with certain malignant diseases, e.g.

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metastatic sarcomas, relapsed brain tumors and other high-risk refractory and/or relapsed malignancies, remains poor [1]. Therapy intensification beyond the standard maximum-tolerated doses (MTD) through the dose escalation approach has not yielded substantial evidence of improved therapeutic response and survival. Only in a subset of patients, such as high-risk neuroblastomas, high-dose therapies have been associated with improved outcome [2]. The introduction of novel therapies, such as monoclonal antibodies, tyrosine kinase inhibitors or other 'targeted' therapies, have, as of yet, not reached the expected therapeutic benefit [1, 3, 4]. At present, these novel treatment approaches still continue to be associated with limited efficacy and sometimes, but less frequently, with significant toxicities [5, 6]. Novel therapeutic approaches for direct application are therefore urgently needed to improve the bleak outcome of these patients. As of today, a number of therapeutic agents with previously unexplored potential are readily available [7].

The term metronomic chemotherapy (MC) was first coined by Hanahan [8] in 2000 and was defined as frequent administration of cytotoxic drugs at doses below the MTD but without prolonged drug-free periods. Low-dose metronomic scheduling of drugs previously used for their cytotoxicity [9, 10] has shown an effect in reducing neoangiogenesis necessary for tumor growth and metastasis thus being able to induce tumor dormancy. In contrast to standard-dose chemotherapeutic regimens based on MTD with periods for recovery, MC targets mainly endothelial and other cells within the tumor microenvironment. These normal host cells of the tumor microenvironment can still be responsive to chemotherapeutic agents, and even very low doses appear to provide effective control of tumor growth with minimal toxicity [7, 9, 10].

The results of metronomic studies showed that this treatment is feasible with surprisingly promising results in some children [11–16]. The MC philosophy and drug usage in pediatric oncology have been reviewed recently [4]. In 2006, we published our first experience with low-dose MC using a protocol named COMBAT (Combined Oral Maintenance Biodifferentiating and Antiangiogenic Therapy), where we summarized our clinical results on 22 heavily pretreated children with relapsed solid tumors [11]. In this protocol, named COMBAT I, we used celecoxib at 200 mg/m^2, etoposide at 25 mg/m^2, temozolomide at 60 mg/m^2 and isotretinoin at 100 mg/m^2. Nine of 14 children (64%) with progressive disease (PD) achieved clinical response. Oral medication was well tolerated with minimal toxicity. Encouraged by these results, we decided to continue the same strategy using modified versions named COMBAT II and COMBAT III. Here, we report on our experience with the use of COMBAT I–III in 74 children with relapsed malignancies treated at three pediatric cancer centers in Europe, for whom the complete data set was available.

Patients and Methods

Patients and Treatment

During the period from December 2004 to September 2010, 74 pediatric patients from three centers (Brno, Czech Republic; Kosice, Slovakia, and Marseille, France), for whom no other higher-priority treatment was available, received MC according to the COMBAT protocol. In accordance with the guidelines of the participating institutions, informed consent was obtained from their parents or legal guardians. COMBAT was offered to 83 patients; 80 consented and 74 patients were available for evaluation with complete datasets. The Institutional Review Board of the University Hospital Brno approved this study as treatment recommendation with data registry.

Seventy-four patients received 77 complete treatments of COMBAT I, II or III; 3 of them were re-challenged with a higher-level protocol.

Eligibility Criteria

- Patients with progressive, relapsed solid tumors
- Patients with documented viable persistent tumors after completion of the original, planned protocol treatment
- Patients at high risk for relapse in whom the 2nd and higher complete response (CR) was achieved by local treatments (surgery or radiotherapy) with/without chemotherapy
- Patients with very high probability of relapse or progression received COMBAT as maintenance after the 1st CR
- Life expectancy of >6 weeks
- Karnofsky/Lansky score >50
- Adequate bone marrow function (ANC >750/mm^3/platelets >75,000/mm^3)
- Adequate hepatic function (alanine aminotransferase <2 times normal)
- Normal renal function (plasma creatinine and/or glomerular filtration rate)

Treatment Details

Duration of COMBAT I treatment was 1 year; patients receiving the COMBAT II or III protocol were offered 2 years of chemotherapy in total. Each year consisted of 4 cycles lasting 78 days each. The COMBAT II protocol contained two strata – the first for soft tissue sarcomas and Ewing sarcomas using the EpSSG (European Soft Tissue Sarcoma Study Group) backbone [16] referred to as COMBAT IIS and the second stratum for all other tumor types based on our original COMBAT I (fig. 1–5).

The differences between the COMBAT II and the COMBAT I scheme were:

(1) Longer duration of chemotherapy exposure (2 years of chemotherapy in total) is based on available published literature [17–19] suggesting clinical benefit of longer chemotherapy duration while respecting acceptable cumulative doses of alkylating agents and etoposide.

Agent	Dose	Route	Schedule	Cycle length
Celecoxib (Celebrex®)	200 mg/m²/day	p.o. divided b.i.d.	days 1–77	11 weeks (77 days)
Etoposide (VePesid®, Lastet®)	25 mg/m²/day	p.o. in the a.m.	days 1–21	
Temozolomide (Temodal®)	60 mg/m²/day	p.o. in the a.m.	days 36–77	
Isotretinoin (Roaccutane®)	100 mg/m²/day	p.o. divided b.i.d.	days 1–14, 29–42, 57–70	

Fig. 1. Protocol COMBAT I.

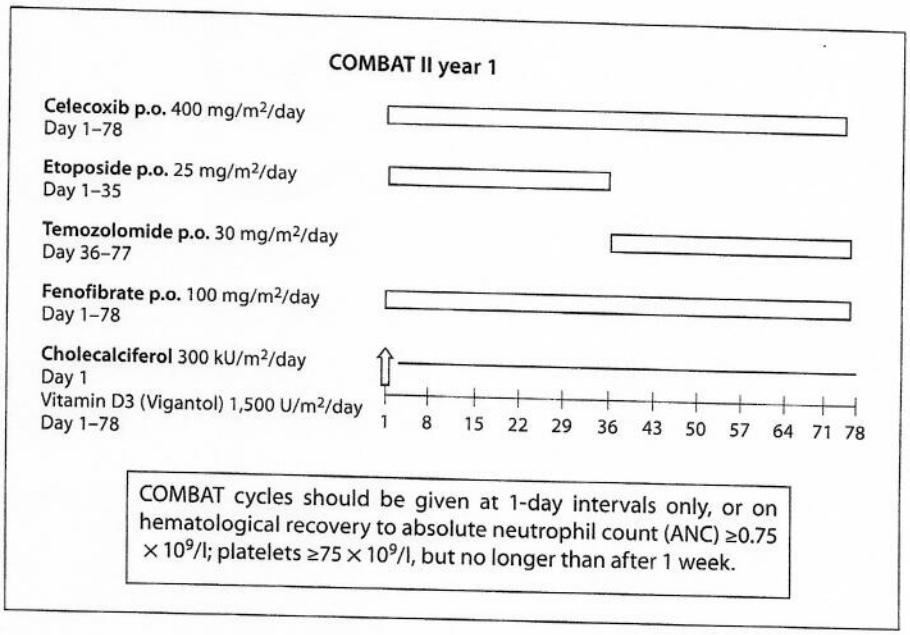


Fig. 2. Protocol COMBAT II year 1.

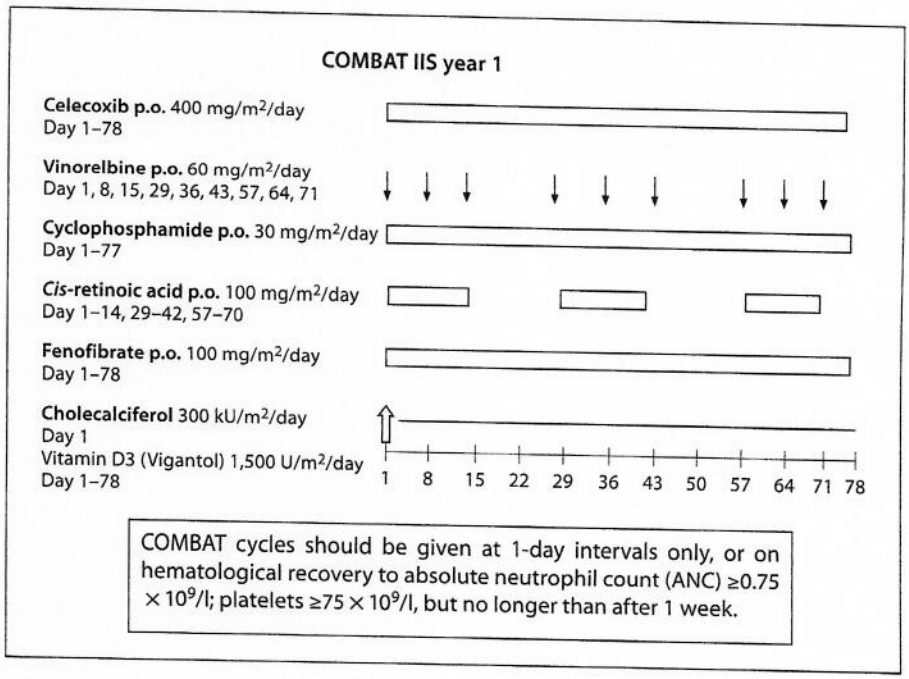


Fig. 3. Protocol COMBAT IIS year 1.

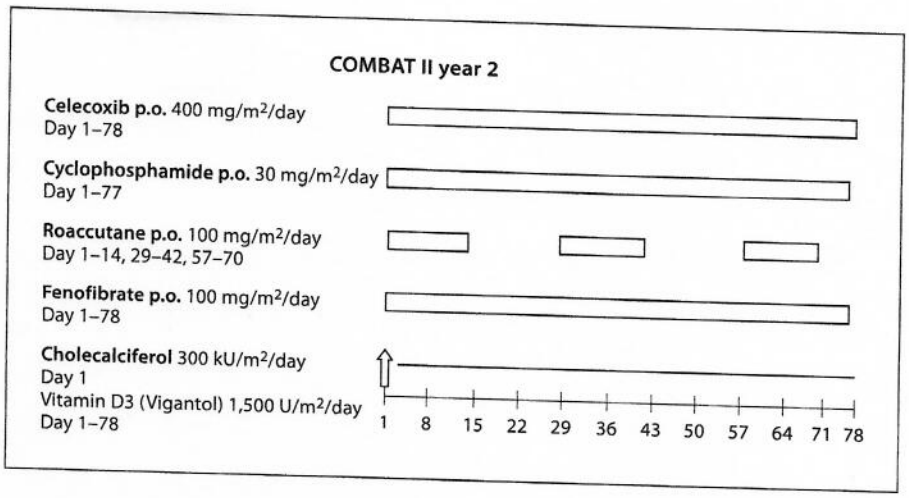


Fig. 4. Protocol COMBAT II year 2.

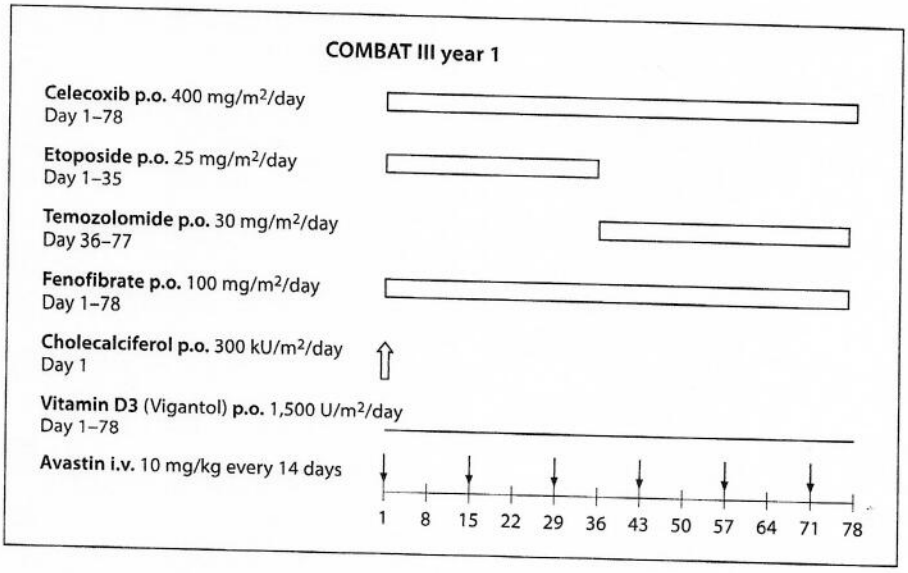


Fig. 5. Protocol COMBAT III year 1.

(2) Addition of fenofibrate is based on the recent publications about its anti-angiogenic and other anti-cancer properties [20, 21]. Fenofibrate is a synthetic ligand for the nuclear receptor peroxisome proliferator-activated receptor (PPAR)α. It is commonly used for the treatment of hyperlipidemia due to its strong lipid-lowering properties. PPARα is not only expressed in tumor cells but also in endothelial and inflammatory cells, and it can also display anticancer properties [20, 21].

(3) Supplementation with vitamin D_3 is based on adult epidemiological data demonstrating that deficiency in this vitamin may be related to an increased incidence and decreased survival of various cancer types [22].

(4) In the non-sarcomatous stratum, we continued using the same cytostatic drugs (etoposide/temozolomide), but we lowered the dose of temozolomide to one half of the previous dose to avoid hematological toxicity, bearing the risks of a cumulative dose in mind. T_{reg} depletion induced by a decreased dose of metronomic temozolomide in a rat model of glioma further supported the dose reduction [23].

(5) We did not use isotretinoin in this stratum during the 1st year of treatment because of its cutaneous toxicity.

In COMBAT IIS, we offered the patients with soft tissue and Ewing sarcomas the same backbone (vinorelbine + cyclophosphamide) as in the recent EpSSG protocol based on a report by Casanova et al. [16], together with COX-2 inhibitors, fenofibrate, vitamin D and retinoic acid. This approach may enable the comparison of chemotherapy alone in EpSSG and COMBAT IIS.

Treatment schemes were used sequentially, reflecting the development of treatment protocols over time. We report on 22 patients treated with the original COMBAT I (also reported for a shorter follow-up [11]) and on 52 newly enrolled patients.

Patients progressing on COMBAT II and patients newly enrolled from August 2009 were offered the COMBAT II protocol

(i.e. COMBAT III) combined with bevacizumab (Avastin; 10 mg/kg i.v. every 2 weeks), a newly available anti-angiogenic drug [17]. After 1 year of COMBAT III, patients received the same drug combination as in COMBAT II for 1 year (2nd year). Isotretinoin was reinstituted during the 2nd year of treatment. Patients were subjected to local therapy, either surgery or radiotherapy, based on individual patient and clinical assessment.

Our general strategy was rather to reduce chemotherapy doses if needed (to decrease toxicity, for example) to avoid chemotherapy discontinuation.

Evaluation of Response and Toxicity

We used RECIST (Response Evaluation Criteria in Solid Tumors) from the National Cancer Institute (table 1) except for CNS tumors. For CNS tumors, only lesions ≥1 cm were set as measurable disease, and a total of 5 such lesions per organ (10 lesions in total) were selected as target lesions (table 2).

The term 'very good partial response (PR)' was used for children with neuroblastoma defined by the International Neuroblastoma Staging System. Toxicities were graded according to the National Cancer Institute (NCI) Common Toxicity Criteria (CTC), version 2; only cheilitis was evaluated according to CTCAE (Common Terminology Criteria for Adverse Events), version 3.

During COMBAT therapy, all patients had regular physical examinations and complete blood cell counts, and assessments of hepatic and renal function were carried out every 2 weeks. Imaging studies to evaluate disease status were performed every 2 cycles or as clinically indicated. Clinical benefit was defined by the number of patients who achieved CR, PR or stable disease (SD) 6 months after starting COMBAT treatment.

The Institutional Review Board of the University Hospital Brno approved this study.

Statistics

Summary statistics was used for sample description. Survival times were computed based on Kaplan-Meier estimates of survival function. Differences in survival times were tested using either the log-rank test for two-group comparisons or a generalized version of Gehan's generalized Wilcoxon test for multiple-group comparisons. All analyses were done using Statistica for Windows 9. Due to the relatively short follow-up time, 2-year survival proportions were given. For event-free survival analyses, the first signs of progression or death were considered an event. $\alpha < 0.05$ was the level indicating statistical significance.

Results

Patient Characteristics

There were slightly more males (47 males, 64%) than females in the total group of 74 children. Mean age was 10 years (SD 6.8 years), varying between 0.9 and 32.4 years. COMBAT I was administered to 28 patients, COMBAT II to 44 patients, and the 5 patients who had a long enough follow-up for evaluation received COMBAT III (tables 3, 4).

A substantially heterogeneous and mostly heavily pretreated patient population was descriptively divided into three different histogenetic groups (low-grade tumors, LG, non-sarcomatous embryonal tumors, EN, and other high-grade tumors, HG) to enable statistical evaluation. The patient characteristics are reported in table 3.

Table 1. RECIST

CR	– complete disappearance of disease according to MRI/CT
PR	– >30% reduction in the longest diameter
SD	– neither CR, PR nor PD
PD	– >20% increase in the longest diameter of the target lesion

Table 2. Response evaluation criteria in CNS tumors

CR	– complete clearing of radiographic disease for at least 4 weeks
PR	– >50% decrease in the sum of the products of all two-dimensional masses for a minimum of 4 weeks
SD	– neither CR, PR nor PD
PD	– >25% increase in the size of a measurable lesion on imaging studies or new lesions on CT/MRI

Most of the patients entered the study with PD or relapsed disease (43 patients, 56%). Fifteen children had a PR (19%), 4 patients SD (5%) and 15 patients were in CR (19%). Nine of them were in ≥2nd CR induced by local treatments.

Patients with a 1st CR were:

- Patients with slowly responding metastatic medulloblastoma (MBL)
- Patients with poor histological response to neoadjuvant treatment and major dose reductions and delays due to treatment-related toxicities (n = 2: 1 with HG osteosarcoma and 1 with Ewing sarcoma)
- Patients with metastatic alveolar rhabdomyosarcoma (n = 3) who did not tolerate standard MTD-based regimens were offered MC as maintenance treatment

Sixty-two patients (81%) had measurable disease. Local treatment (surgery/radiotherapy) was used in 12 patients before and during COMBAT treatment. Median follow-up lasted 14.3 months (range: 1.3–64 months).

Treatment Outcome

The 2-year OS in the 74 patients was 43.1% (median: 15.4 months; fig. 6). Of 62 patients with initially measurable disease, 25 patients improved; those who were progressing did not worsen during the entire follow-up period (40%). Clinical benefit (defined by the number of patients with measurable disease who achieved CR, PR or SD 6 months after starting COMBAT) was 23%. Addi-

Table 3. Patient characteristics at baseline

Diagnostic group		n	Age, years mean ± SD	Males n (%)	Previous therapy			Disease status prior to COMBAT		
					HDT with SCT	standard CHT	RT	CR	PR/SD	PD
EN	MBL	13	9.9 ± 5.80	9 (69)	12	0	13	5	3	5
	Neuroblastoma	11	6.6 ± 6.23	7 (64)	6	0	9	0	0	11
	Other embryonal, non-sarcomatous	9	6.4 ± 5.62	6 (67)	5	1	7	0	4	5
HG	Sarcoma	17	14.1 ± 4.66	7 (41)	8	2	13	8	3	6
	Other	18	10.2 ± 5.80	13 (72)	1	9	12	2	7	9
LG		9	10.2 ± 11.16	8 (89)	1	3	5	0	2	7

HDT = High-dose therapy; RT = radiotherapy; CHT = chemotherapy.

Table 4. Patient response to metronomic chemotherapy

Diagnostic group		n	Median survival months	Events deaths n (%)
EN	MBL	13	40.8	9 (69)
	Neuroblastoma	11	21.7	10 (91)
	Other embryonal, non-sarcomatous	9	30.7	8 (88)
HG	Sarcoma	17	35.2	13 (76)
	Other	18	60.9	11 (61)
LG		9	–	1 (11)

tional 9 patients responded after the first 6 months of COMBAT treatment. No statistically significant effect of gender/age on patient outcome was evident. However, LG patients lived significantly longer ($p = 0.004$) than HG patients (fig. 7). There was no difference documented for embryonal tumors compared to other, non-embryonal HG (fig. 7). Similarly, patients without PD at baseline survived significantly longer ($p = 0.011$). Regarding survival, three clearly distinctive ($p < 0.001$) groups of patients were obtained: LG patients and HG patients (including embryonal tumors) without and with PD with 2-year OS amounting to 88.9, 53.8 and 22.2%, respectively (fig. 7).

Addition of concomitant local therapy (e.g. surgery/radiotherapy) in addition to the above-described COMBAT regimens had no statistically significant effect on OS or event-free survival, but the sample size may have been too small to detect an effect (fig. 8). Considering the COMBAT regimens, outcome was superior in patients treated with the newer COMBAT regimens (II or III; $p < 0.005$). However, the follow-up of patients on COMBAT III is still quite short compared with the original cohort on COMBAT I.

Of the entire cohort (74 children), 50 patients (68%) died during the follow-up and 24 survived: 6 (8%) PD patients, 7 (9%) PR/SD patients and 11 (15%) CR patients. Median time to response (CR/PR) in patients with measurable disease was 6 months.

Detailed data analysis, including radiological imaging, was available for 12 children with MBL/primitive neuroectodermal tumor (PNET) who had relapsed following high-dose therapy with stem cell support; they represented the cohort of MBL/PNET patients with the worst prognosis. Two-year OS in these COMBAT-treated patients was 33%, including 2 long-term survivors (64 and 54 months). Nine patients were evaluated for the pattern of their 2nd progression after/during COMBAT treatment. Two of these 9 patients started COMBAT during their 2nd CR after surgery and also relapsed during MC with measurable parenchymal lesions and leptomeningeal disease.

Two other patients had measurable parenchymal lesions at MC initiation without cytological CSF involvement or documented leptomeningeal disease on MRI. One of them is a long-term survivor. The remaining 5 out of 9 patients with 2nd progression had evidence of leptomeningeal disease while entering COMBAT. Three continued to progress despite COMBAT and 2 achieved

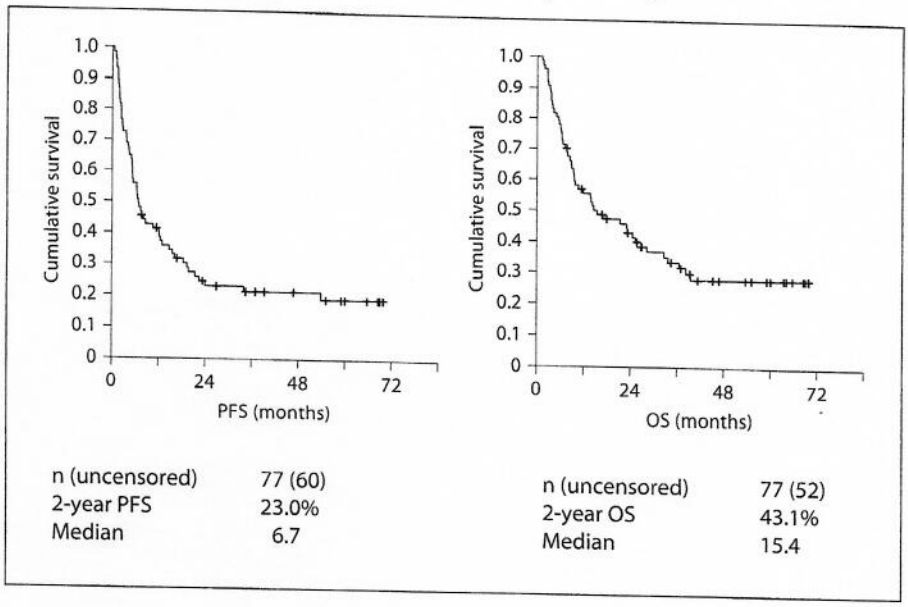


Fig. 6. Progression-free survival/overall survival, all patients.

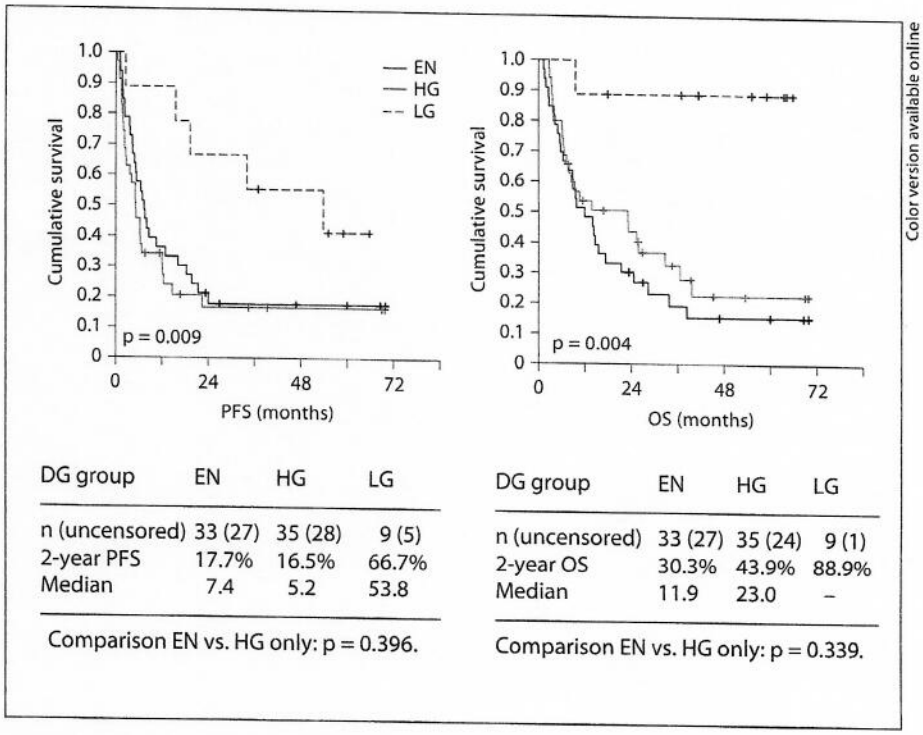
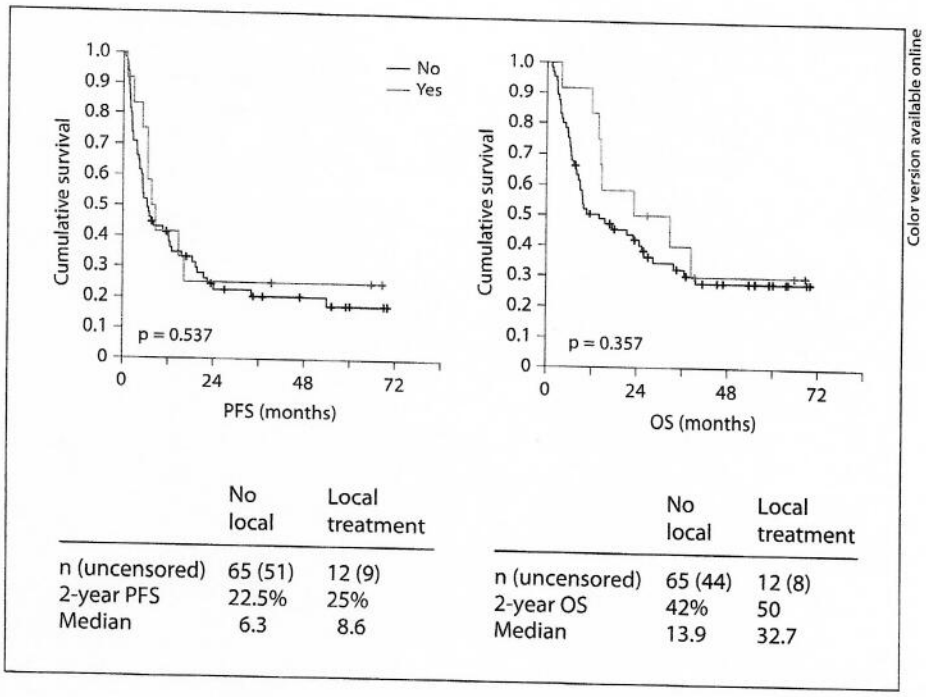


Fig. 7. Progression-free survival/overall survival, patients by diagnostic groups.

CR. One of the patients in CR following COMBAT later developed PD shortly after the planned withdrawal of COMBAT, and the other developed secondary acute myeloid leukemia (AML) following intensive chemotherapy and lives after unrelated bone marrow transplantation in CR of either MBL or AML [18].

Of 12 patients with progressive neuroblastoma, only 2 experienced durable responses: 1 PR in a patient with progression-free survival (PFS) for 63 months and 1 with 2nd very good PR at the last 12-month follow-up. The remaining 9 patients showed temporary responses or disease stabilization (8 of 9), with marked reduction in the size of the soft tissue mass. However, further progression



	No local	Local treatment
n (uncensored)	65 (51)	12 (9)
2-year PFS	22.5%	25%
Median	6.3	8.6

	No local	Local treatment
n (uncensored)	65 (44)	12 (8)
2-year OS	42%	50
Median	13.9	32.7

Fig. 8. Effect of local treatment.

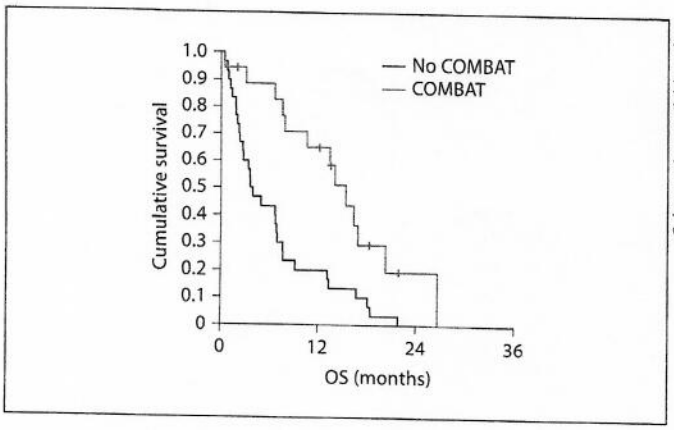


Fig. 9. Comparison of the COMBAT regimen with historical control group treated with MTD-based chemotherapy. COMBAT n = 18, non-COMBAT n = 30. Time to progression in COMBAT: 18.1 months. Time to progression in a control group: 11.7 months (p = 0.015). Median OS COMBAT: 15.4 months. Median OS in control group: 3.9 months (p = 0.001).

occurred despite COMBAT especially in children with bone marrow involvement, or after previous therapeutic MIBG, with a median time to death of 7.2 months only for those 9 who progressed.

Similarly, for patients with relapsed or progressive HG sarcomas, COMBAT did not induce any long-term responses, but a palliative effect of MC was evident. Eighteen patients with HG sarcoma treated on COMBAT I and II regimens from 2004 to 2009 (without bevacizumab) were compared to a historical control group of 30 patients (8 with soft tissue sarcoma, 9 with Ewing sarcoma, 13 with osteosarcoma, and 15 with local and 15 with metastatic progression) treated with MTD-based chemotherapy from 1999 to 2004. Time to progression (TTP) in the COMBAT and historical control groups was 18.1 and 11.7 months, respectively (p = 0.015). Median OS in the patients treated with COMBAT was 15.4 months; median OS in the control group was 3.9 months (p = 0.001; fig. 9). Of interest, in 2 patients with metastatic HG sarcomas (Ewing sarcoma and alveolar rhabdomyosarcoma progressing on COMBAT III), durable responses were induced by MC combined with mTOR inhibitors.

Eight of 9 children with progressive LG (LG glioma, LGG, giant bone cell tumors with lung metastases and LG sarcomas) responded to therapy, only 1 of 10 died from tumor progression, the remaining children are alive with a median PFS of 33 months, and duration of clinical benefit after MC is longer compared to previous treatments. Moreover, 2 patients with progressive lung metastases of the giant cell tumor of bone experienced prolonged disease stabilization on MC (PFS for 34 and 15 months, respectively).

Table 5. Worst degree of COMBAT-related toxicity reported among 74 patients evaluable for toxicity across all courses (NCI-CTC, version 2.0)

Toxicity	None	1	2	3	4	Unknown
Hemoglobin	0	22	31	19	2	0
WBC	0	16	28	23	7	0
ANC	0	15	29	21	9	0
Platelets	0	11	32	22	9	0
Febrile neutropenia/sepsis/septic shock	70	0	0	4	0	0
Other infections	54	11	9	0	0	0
Neurotoxicity	58	9	7	0	0	0
Hypokalemia	61	7	6	0	0	0
Hyponatremia	64	6	4	0	0	0
Hyperglycemia	59	9	6	0	0	0
ALT	24	32	12	6	0	0
Bilirubin	22	33	11	8	0	0
Nausea	41	21	12	0	0	0
Vomiting	54	13	7	0	0	0
Diarrhea	64	10	0	0	0	0
Constipation	55	11	8	0	0	0
Stomatitis	57	12	5	0	0	0
Cheilitis (CTCAE, version 3.0)	3	22	33	16	NA	NA
Partial thromboplastin time	56	11	7	0	0	0
Prothrombin time (as INR)	49	14	11	0	0	0
Hypertriglyceridemia	23	26	19	6	0	0

Three children with hepatocellular carcinoma, 2 after marginal resection and 1 after complete resection, remained free of disease with follow-ups of 36, 13 and 13 months, respectively. Patients were not included in statistical analysis because their treatment was started after achieving 1st CR by surgery.

Toxicity Profile

COMBAT-related toxicities observed during the treatment courses are listed in table 5. The overall treatment tolerance was very good; treatment was administered on an outpatient basis, and there was no treatment-related death or grade III/IV non-hematological toxicity except for skin or hepatic toxicity. Other observed toxicities were rather attributable to disease progression (for example seizures in progressive supratentorial PNET) than to MC.

Regarding non-hematological toxicity, only 8 children (11%) showed hepatic toxicity grade 3. The only other grade 3 non-hematological toxicity was cheilitis in 16 children (22%) during *cis*-retinoic acid treatment. One patient with early progressing metastatic MBL treated with myeloablative thiotepa-based chemotherapy, autologous stem cell transplant (SCT) and COMBAT as maintenance developed secondary AML 2 years after autologous SCT and 1 year after MC cessation. He underwent successful allogeneic bone marrow transplantation, and CR was obtained to both MBL and secondary AML, too.

Discussion

Our results are in line with previous reports showing some degree of response to MC in children with refractory or relapsing disease combined with a very favorable toxicity profile. In this study, the response rates varied with tumor type and even within the same histology based on different parameters. For instance, children with neuroblastoma showing slow progression on MIBG or slowly raising catecholamines (mainly soft tissue cancer) benefited from COMBAT, while rapidly progressing tumors, especially those with bone marrow involvement, did not. However, for responding neuroblastoma patients, the COMBAT regimen could serve as an effective, bridging cytoreductive regimen with low toxicity profile, enabling thus second high-dose therapy, another type of consolidation or therapy continuation. Interestingly, LG benefited most from MC; therefore, metronomic ap-

proaches for those tumors probably deserve further clinical assessment.

We still do not know which patients are the best candidates for low-dose MC. Clearly, patients with galloping, progressive high-grade tumors such as relapsed neuroblastoma following high-dose therapy (especially with bone marrow involvement) are not the best candidates for MC because of tumor aggressiveness. The nature of the treatment, i.e. the induction of dormancy via inhibition of the inflammatory and pro-angiogenic stromal response by the metronomic scheduling, may be precluding the use of this therapy in aggressive disease, although responses were observed in atypical teratoid rhabdoid tumor or MBL with both a metronomic regimen similar to COMBAT [14] and a multi-targeted multidrug MC regimen [17]. Usually, best results with MC lead to stable disease [11, 14, 15, 19] very likely through 'maintenance or re-induction of dormancy' [4, 19] or alternatively by further slowing down a slowly advancing tumor. Contrary to the commonly held beliefs, the tumors that are most responsive to anti-angiogenic therapy usually grow slowly like LGG or desmoid tumors [24].

The question of whether MC may be used as maintenance therapy in selected very-high-risk subsets, or whether it should be used for overt relapses, seems to be answered by the finding that 10 of 11 children with relapsed neuroblastoma continued to progress following the institution of COMBAT. In contrast, children with slowly growing tumors such as LGG, giant cell tumor of bone or slowly progressing neuroblastomas benefited clinically. Similarly, when retinoic acid or anti-GD2 antibodies are used as maintenance treatment, they contributed to treatment success while their efficacy in overt relapse is much less apparent [27, 28]. Furthermore, MC might be considered an attractive option for lower-income countries or in centers with limited access to 'modern or innovative' therapies [25, 26].

For patients with giant cell of bone and progressive lung metastases, one may consider the recently registered denosumab as higher-priority treatment; however, denosumab use for this condition is still off-label in many countries thus rendering MC still worth to consider.

The other unresolved issue is the accurate endpoint of MC in clinical trials. Prolonged time intervals to response and the very good quality of life in MC-treated patients support the quite new idea of 'living with the tumor' [19, 29]. Dealing with tumors like with any other chronic disease introduces a new kind of problem – is stabilization of disease enough for the patient and, consequently, can this be considered as one of the possible endpoints of MC? We believe that the answer to this clinically important question is yes.

Considering specific diagnoses, it seems that patients with hepatocellular carcinoma after surgery may benefit from MC as the usual long-term OS is only about 25%. LG patients progressing on standard chemotherapy protocols may also benefit from MC. While analyzing MBL/PNET patients, the leptomeningeal pattern of progression on COMBAT in our series may raise the question of how to better address leptomeningeal disease, possibly combining intrathecal chemotherapy with MC for relapsed embryonal CNS tumors in pediatric patients [17, 30].

In the palliative setting, it is sometimes very difficult to stop seemingly or truly effective treatments, especially when parents or the patient himself prefer to stay on at least some form of antineoplastic treatment. Since COMBAT is given on an outpatient basis with minimal acute toxicity, which thus preserves a high quality of life, only few patients are willing to discontinue therapy and re-evaluate. According to published results, time to response in adult trials or case reports is several months, but pediatric data on this topic are mostly lacking [31, 32]. Our data suggest that some patients might benefit from prolonged therapy, which is supported by the results on our LG patients (sarcomas and LGG) or a previously published case with MBL, in whom best response lasted over 600 days and progression advanced shortly after MC withdrawal [18].

Statistical consideration in pediatric metronomic studies is also very complex, because the population consists of already heavily pretreated children with different types of solid tumors and a large array of different therapies. It is extremely difficult to conduct randomized trials in this group of desperate patients being at very high risk of relapse or progression. MC duration also remains empirical, keeping in mind individual tolerance, efficacy and cumulative doses. One possible way to assess the role of MC could be comparisons with large historical control groups [34, 35], as presented here for HG sarcoma patients (fig. 9). We are aware of the bias associated with historical control, but this strategy may nevertheless be useful to gain the first insight into clinical activity. Another possible way could be to use TTP, i.e. comparing TTP while the patient is receiving the therapy immediately before the treatment of interest versus TTP while the patient is under experimental treatment [33, 35].

A methodological challenge also relies in the fact that, as mentioned earlier for neuroblastoma, a treatment applied as a salvage treatment might exert different effects as a maintenance treatment and vice versa. This question

is being tested within a randomized study with the use of the navelbine-low-dose cyclophosphamide combination [16] in high-grade rhabdomyosarcoma by the International Society of Pediatric Oncology group.

Conclusions

As illustrated by our experience with COMBAT, MC delivered promising results in pediatric oncology. The existence of children living with some of the most debilitating diseases who may turn to long-term survivors when treated with COMBAT combined with the appearance of late responses paves the way for a switch in the intend of treatment from palliative only to rather curative, at least in some patients. Alternatively, introducing MC at earlier phases of antitumor treatment may allow further exploitation of the potential of low-dose MC, for instance as maintenance therapy.

However, many questions still remain: What are the indications? How to find out accurate dosing and combinations of drugs? What are the relevant endpoints and biomarkers for these studies? How long should we treat patients? Apparently, a *'one COMBAT fits all'* scheme will not be clinically useful in general. To find answers and cooperate at the international level, a metronomic network was established and two metronomic therapy meetings took place in Brno (2008) and Marseille (2010) [34]. The goal of the network is to attract clinical interest and facilitate further disease-oriented metronomic therapy-based clinical research [34].

Disclosure Statement

The authors have no conflicts of interest.

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CASE REPORT Open Access

Case report: rapid and durable response to PDGFR targeted therapy in a child with refractory multiple infantile myofibromatosis and a heterozygous germline mutation of the *PDGFRB* gene

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Abstract

Background: Infantile myofibromatosis belongs to a family of soft tissue tumors. The majority of these tumors have benign behavior but resistant and malignant courses are known, namely in tumors with visceral involvement. The standard of care is surgical resection. Observations suggest that low dose chemotherapy is beneficial. The treatment of resistant or relapsed patients with multifocal disease remains challenging. Patients that harbor an actionable mutation in the kinase domain are potential subjects for targeted tyrosine kinase inhibitor therapy.

Case presentation: An infant boy with inborn generalized infantile myofibromatosis that included bone, intracranial, soft tissue and visceral involvement was treated according to recent recommendations with low dose chemotherapy. The presence of a partial but temporary response led to a second line of treatment with six cycles of chemotherapy, which achieved a partial response again but was followed by severe toxicity. The generalized progression of the disease was observed later. Genetic analyses were performed and revealed a *PDGFRB* gene c. 1681C>A missense heterozygous germline mutation, high PDGFRβ phosphokinase activity within the tumor and the heterozygous germline Slavic Nijmegen breakage syndrome 657del5 mutation in the *NBN* gene. Targeted treatment with sunitinib, the PDGFRβ inhibitor, plus low dose vinblastine led to an unexpected and durable response without toxicities or limitations to daily life activities. The presence of the Slavic *NBN* gene mutation limited standard chemotherapy dosing due to severe toxicities. Sister of the patient suffred from skull base tumor with same genotype and histology. The same targeted therapy led to similar quick and durable response.

Conclusion: Progressive and resistant incurable infantile myofibromatosis can be successfully treated with the new approach described herein. Detailed insights into the biology of the patient's tumor and genome are necessary to understand the mechanisms of activity of less toxic and effective drugs except for up to date population-based chemotherapy regimens.

Keywords: Infantile myofibromatosis, Tyrosine kinase inhibitor, PDGFR, Chemotherapy, Theranostics, Case report

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Background

The family of fibroblastic-myofibroblastic tumors consists of more than 30 distinguished entities, such as inflammatory myofibroblastic tumor (IMT), aggressive fibromatosis and infantile myofibromatosis (IM). These tumors have uncertain biologic behaviors that range from low grade, locally aggressive and rarely metastasizing to a highly aggressive course that eventually evolves to a true high-grade sarcoma after recurrences. IM is a rare tumor that affects infants with a median age of 3 months; approximately 100 solitary lesion cases have been published in the literature during the past decade [1]. Soft tissue lesions of IM can arise at any time during life and, intriguingly, can regress spontaneously. However, visceral lesions are associated with high morbidity and mortality. The standard of care is the surgical resection of a single lesion. Multiple lesions and surgically unresectable lesions could be treated with anti-inflammatory drugs, interferon alpha, or distinct chemotherapeutic regimens that are based on low dose metronomic or maximum tolerated doses (MTD) of chemotherapeutics, such as the vinca alkaloids vincristine, vinorelbine and vinblastine; the alkylating agents cyclophosphamide and ifosfamide; or others, such as actinomycine D, doxorubicin or methotrexate [2–4]. The results of such treatments are under investigation in ongoing observational clinical trials of cooperative groups, such as European Soft Tissue Sarcoma Study Group (EpSSG) or Children's Oncology Group (COG). Several studies of desmoid-type fibromatosis with response rates of 33–49% were reviewed elsewhere [4]. Nevertheless, the treatment of resistant patients, particularly those with visceral involvement, remains challenging.

For patients with progressive disease after MTD based chemotherapy, there are no established standards of care, and these patients are, thus, subjected to experimental treatments. One of the most promising agents with proven activity for IMT is the ALK tyrosine kinase inhibitor crizotinib [5]. Patients with ALK rearrangement are reportedly rapidly responding to crizotinib, but those without the detected fusion are not [5]. A recent work by Lovly et al. on IMTs revealed multiple fusion partners of ALK, and newly reported ROS1 and PDGFRβ fusions with projected TKI sensitivity were demonstrated in a patient with an ROS1 fusion [6]. Similar to IMTs, IMs may harbor missense mutations in the PDGFRβ kinase that constitutively alter PDGFR activity. Moreover, in several families, the c.1681C>T (p.Arg561Cys) mutation in the *PDGFRB* gene was found to cause familial infantile myofibromatosis [7]. A phase II study of sunitinib in 19 patients with aggressive fibromatosis has been published and described a 26.3% overall response, but the analysis of the kinase pathway was lacking [8]. A case report of aggressive fibromatosis that favored the PDGFRβ inhibitor sunitinib against imatinib was published that described a good response with sunitinib which was interrupted after 13 months and substituted by imatinib. But reactivation of painful lesions occurred within several days and re-growth of aggressive fibromatosis led to successful re-treatment with sunitinib [9].

Herein, we report the case of a patient with refractory multiple infantile myofibromatosis who was confirmed to harbor the *PDGFRB* germline mutation and who responded well to treatment with the PDGFRβ tyrosine kinase inhibitor sunitinib.

Case presentation

The newborn boy with microtia and meatal atresia and with family history of two spontaneous missed abortions and myofibroblastic lesions with spontaneous regression in his older sister and father, was diagnosed with generalized myofibromatosis that affected the calva and radius bones, the spleen and subcutaneous tissue of face, the head, inguina and arm. Histopathology, with regard to the family history, revealed the presence of infantile familial myofibromatosis. Immunohistochemistry (ICH) and FISH did not reveal any pathological staining for ALK. The patient was treated according to the EpSSG 2005 observational trial recommendation with the metronomic vinblastine/methotrexate combination, which was expected to be less toxic than MTD based regimens. Despite this, severe neutropenia had been observed; therefore, a dose reduction was necessary down to 10%/30% of the original doses of vinblastine/methotrexate, respectively. The therapy was stopped after 8 weeks due to clearly progressive disease in the soft tissues and in the spleen and with the appearance of new FDG PET positive lesions in the bones. Thereafter, the standard MTD based therapy with vincristine/actinomycine D/cyclophosphamide – the "VAC" regimen with doses based on body weight (vincristine 0.05 mg/kg, actinomycine D 0.05 mg/kg, cyclophosphamide 50 mg/kg) had been initiated. Such treatment after the second course (the first course was given with a 75% reduction of cyclophosphamide) had led to severe febrile neutropenia, gastrointestinal toxicity with gastric palsy, subileus and bilateral bronchopneumonia. However, a reassessment after those 2 cycles revealed a partial response. Due to the previous toxicity, we decided to substitute vincristine with vinblastine at 10% of the recommended dose and cyclophosphamide at 75% of the recommended dose. The patient received the treatment without dose limiting toxicities up to six cycles and continued to respond. The patient was still in partial remission according to CT and MRI images and the FDG PET of the remaining measurable lesions was negative. Unfortunately, the first follow-up re-assessment confirmed the presence of progressive disease just 3 months

after the last chemotherapy dose and several new lesions were detected in the humerus, head, lungs and skin, and all were FDG-PET positive.

A new biopsy was carried out to obtain tumor tissue for phosphoproteomic analysis of the new lesion. The Human Phospho-RTK Array Kit was used to determine the relative levels of tyrosine phosphorylation of 49 different RTKs. The analysis was performed as previously described [10]. In addition to the antibodies (spotted in duplicate) against individual RTKs, each membrane contained three positive reference double spots and one negative control that was also spotted in duplicate and contained phosphate-buffered saline only. Furthermore, we also performed the following negative control experiment in each run: the membrane treated with lysis buffer only (without protein lysate) to ensure the specificity of the spotted antibodies. In such a design, a healthy control sample is not necessary for the determination of the RTK phosphorylation profile of the examined tumor tissue [11–13]. The phosphorylation profile of receptor tyrosine kinases showed that PDGFRβ kinase exhibited the highest level of activity and less intense positivity was observed for EGFR, M-SCFR, Axl and PDGFRα (Fig. 1). Targeted DNA analysis of the *PDGFRB* gene and next generation sequencing (NGS) were performed on genomic DNA from peripheral blood samples. We performed Sanger sequencing of the two *PDGFRB* regions to detect the presence of the c.1978C>A (p.Pro660Thr) and c.1681C>T (p.Arg561Cys) mutations [6] and uncovered a germ-line heterozygous c.1681C>A missense mutation that had previously been shown to be an IM causing mutation [14, 15]. To obtain the complex picture of the genetic background of the case we performed DNA analysis from peripheral blood with the Illumina TruSight Cancer panel, which enabled the sequencing of the hotspots in 94 predisposition cancer genes, according to the standard Illumina protocol (Illumina Inc., USA) and identified the heterozygous Slavic mutation 657del5 in the *NBN* gene of the NBS.

In the meantime, and based on parental request, the patient was observed for the next 4 months. He was doing very well clinically, with a Lansky performance status of 90% and with respect to his treatment history with toxicities after chemotherapy; we did not initiate another chemotherapy regimen but were awaiting the results of genetic analyses, which have revealed potential therapeutic targets. Further follow-up confirmed that the disease continued to progress; several new lesions were detected within the head and the left orbit, a new one was detected in the spine, and the spleen lesion had increased in size.

Due to clear clinical and radiologic progression and new molecular genetic findings, and with respect to the history of the disease, we initiated the single agent *off-label* treatment with sunitinib 12.5 mg once a day. This dose corresponded to 2/3 of the recommended adult dose. An unexpected and dramatic reduction of the palpable soft tissue and bony lesions on the head was observed during the 4 weeks of treatment with the single agent sunitinib. An MR scan confirmed the regression of intracranial and intraorbital lesions as well (Figs. 2, 3 and 4). However, this dosing schedule led to grade 3–4 neutropenia, and the drug was stopped for 4 days. After only 4 days, we could observe the reactivation of the skin and soft tissue lesions; therefore, the sunitinib was given at the same dose every other day. Reactivated reddish swollen and painful sentinel lesions responded again to lower doses of sunitinib, but three more weeks of reduced doses of the single agent sunitinib did not lead to any further regression of the regressed but still palpable skin lesions. A low dose of vinblastine was added to the sunitinib. The starting vinblastine dose was 2 mg/m^2; however, based on the further hematological

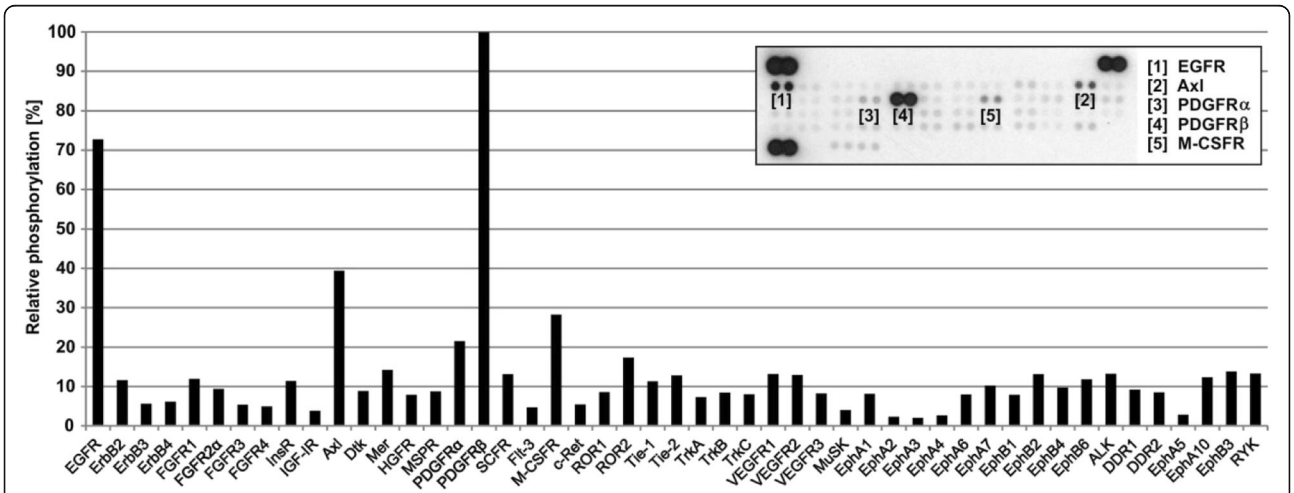

Fig. 1 The relative phosphorylation of kinases in the tumor tissue sample

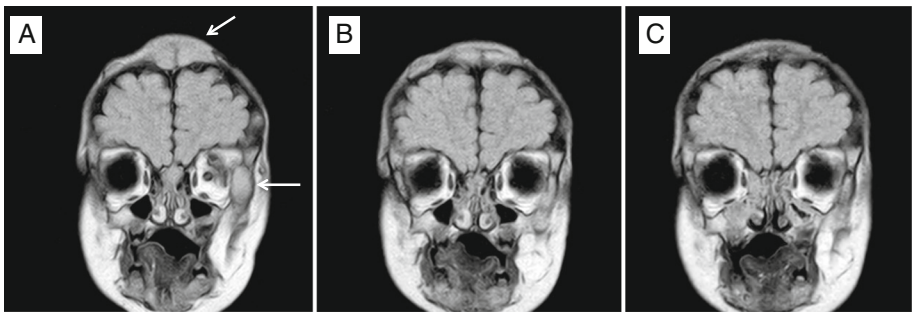

Fig. 2 MRI Frontal view (seq. eFLAIR_long_TR_CLEAR). Two lesions of the left orbit and the skull in the fronto-parietal region (*bars*). **a** Before sunitinib treatment. **b** Day + 56 of sunitinib. **c** Day + 156 of sunitinib

toxicity, the dose was tapered down to a 0.4 mg/m^2 dose once weekly.

An unexpected toxicity of sunitinib occurred after 4 months of treatment when accidental hypoglycemia led to a coma and the patient had to be admitted for glycemia corrections. Thereafter, the parents were educated on regular feeding before sunitinib administration. Further episodes of hypoglycemia were not noted. The patient remained on the treatment paradigm with a marked continuing response with no disease activity 1 year after the initiation of the treatment and without any dose limiting toxicities.

Interestingly, the 8 year old sister of the patient, who had a history of spontaneous regression of subcutaneous lesions, suffered from the symptomatic re-activation of the disease when the patient was receiving treatment. She presented with tumor size of 29 × 24 × 16 mm on the skull base with night pain. Histopathological and detailed mutation analyses found the same IM histopathology and the same genotype in the *PDGFB* and *NBN* genes. As with the index case, the sister is doing well on sunitinib and vinblastine treatment and has exhibited a rapid response. The nigh pain relieved after 2 weeks on sunitinib + vinblastine. Initial tumor volume shrinked by 44% after 97 days of combined treatment without any adverse events requiring reduction of doses. Timeline of both cases is shown on Additional file 1.

Discussion and conclusions

Despite the finding that the patient exhibited a partial response to systemic VAC treatment, the disease continued to progress; moreover, the patient experienced severe, life threatening dose-limiting toxicities.

Inflammatory myofibroblastic tumors that harbor an ALK/ROS1 or PDGFRβ kinase fusion are potentially targetable with TKIs due to the presence of a constitutively active kinase domain that drives cellular proliferation [6, 16]. A response to the ALK inhibitor crizotinib is reported in tumors that harbor any of the ALK kinase fusions. Patients with IMT and ALK negative rearrangements are unlikely to respond to such targeted treatment.

PDGFRB mutations are reported to be involved in the pathogenesis of infantile myofibromatosis in a proposed autosomal dominant pattern with incomplete penetrance and variable expressivity [7]. The missense *PDGFRB* c.1681C>T (R681C) mutation is located in exon 12 and is predicted to decrease the autoinhibition of the JM domain (an autoinhibitory domain that masks the catalytic cleft when the receptor is not bound by its ligand) at baseline, which leads to increased kinase firing and promotes the formation of myofibromas in tissues with high PDGFRβ signaling activity. More recently, it was demonstrated in a cell culture model that the R561C mutation activates signaling pathways that are normally

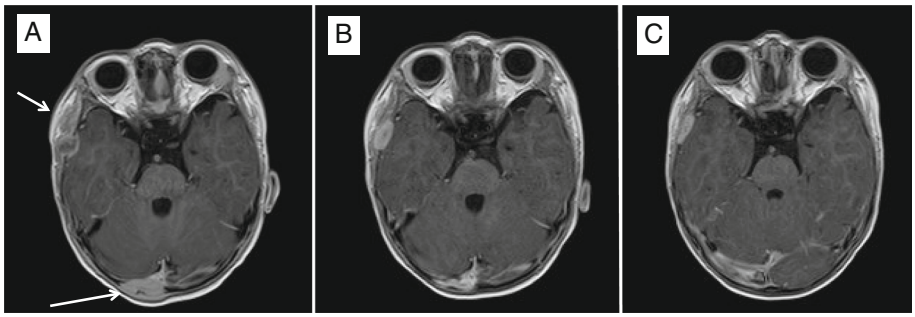

Fig. 3 MRI Axial view (seq. esT1W_3S_FFE post-contrast). Intracranial lesions of the right temporal and right parieto-occipital regions (*bars*). **a** Before sunitinib treatment. **b** Day + 56 of sunitinib. **c** Day + 156 of sunitinib

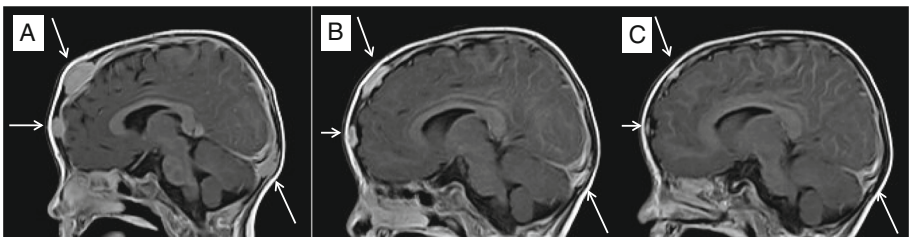

Fig. 4 MRI Sagittal view (seq. esT1W_3S_FFE post-contrast). Frontal and parieto-occipital lesion (*bars*). **a** Before sunitinib treatment. **b** Day + 56 of sunitinib. **c** Day + 156 of sunitinib

activated by the stimulated wild-type PDGFRβ receptor in the absence of PDGF [14]. PDGFR is the immediate NOTCH3 target gene [17]. If these two signaling pathways are linked and the IM disease-causing mutations in either *PDGFRB* or *NOTCH3* are demonstrated to be activating, theoretically, the inhibition of *PDGFRB* or *NOTCH3* would result in a targeted therapeutic strategy [7]. Our case report shows the clinical efficacy of such an approach. Targeted therapy against altered PDGFRβ with a TKIs inhibitor can overcome tumor growth and can lead to tumor shrinkage. Compared to the toxicity of conventional chemotherapy, treatment with sunitinib was tolerated well except for the occurrence of asymptomatic granulocytopenia and one episode of symptomatic hypoglycemia. However, the cessation of the drug lead to increased tumor activity and a decreased drug dose of the single agent sunitinib led to a stable disease only.

The analysis of tumor tissue or a patient's samples and the use of a subsequent results driven treatment provide a new opportunity for personalized medicine as opposed to a population based study. Such treatments are supported by new insights into the molecular pathology of rare diseases, such as IM. A similar strategy would at least justify the *off-label* use of new drugs when the individual tumor biology and data about the safety of such drugs is well defined. TKIs could be an example, as these drugs are not available to orphan disease patients because of the absence of appropriate clinical trials. The careful management and regular observation of the patient is mandatory, however, in situations where standard approaches are either exploited or ineffective or absent, the prudent use of targeted agents based on the mechanism of action might lead to impressive results.

The rapid tumor re-growth that occurred when the patient was off of the sunitinib during the induction treatment indicates that metronomic dosing should be maintained at a lower dose with limited toxicity rather than being interrupted. The successful use of low dose vinblastine that is described here, together with the use of sunitinib at a dose of approximately 1/3 of the usually recommended dose per kg or m^2 in adults, could be at least in part explained by the fact that targeted agents could act as biology response modifiers and lower doses of biological agents and chemotherapy could be nontoxic and advantageous [18, 19]. This theory is supported by our observation of the clear disease progression when sunitinib therapy was interrupted. Regular observations of the patient and preemptive measures such as the after-feeding dosing of sunitinib should be considered during treatment.

The finding of the Slavic mutation of the NBS was noted as accidental during NGS sequencing and the relevance for the disease course is unknown. The toxicity of chemotherapy might be at least in part conditioned by the NBS mutation As known, the intensity of chemotherapy in NBS patients must be adapted to individual risk factors and tolerance. The use of radiomimetics, alkylating agents, and epipodophyllotoxins should be avoided, and the dose of methotrexate should be limited [20].

However, the overall duration of such clinically effective treatment remains speculative, especially in patients with germline mutations. Different approaches that consider cancer to be a chronic disease, such as diabetes, should be considered in instances in which pathogenic germline mutations are in place. Should such targeted agents be maintained for a very long time, e.g., maintenance therapies in childhood acute leukemia, where other mechanisms of action, not only the cytostatic effect are in place? [21]. Should some pulses of targeted agents be considered?

These are only a few of the new questions that arose by the increased availability of diagnostic methods, such as NGS and functional proteomics.

The patients with an orphan disease like IM could benefit from detailed insights into the biology of their tumor and genome. Such approach is necessary to better understand the molecular pattern of disease and mechanisms of action of less toxic and effective drugs except for up to date population-based chemotherapy regimens. Morover, an unexpected finding of germline mutation can be important for treatment decisions. Progressive and resistant incurable infantile myofibromatosis can be successfully treated with the new approach described herein.

Additional file

Additional file 1: Timeline. This file shows timeline of both described cases. (PDF 466 kb)

Abbreviations

ALK: Anaplastic lymphoma kinase; COG: Children's oncology group; EpSSG: European Soft Tissue Sarcoma Study Group; FDG PET: Fluorodeoxyglucose positron emission tomography; FISH: Fluorescent in situ hybridization; IHC: Immunohistochemistry; IM: Infantile myofibromatosis; IMT: Inflammatory myofibroblastic tumor; IVA: Ifosfamide/vincristine/actinomycine D; MRI: Magnetic resonance imaging; MTD: Maximum tolerated doses; MTX: Methotrexate; NBS: Nijmegen breakage syndrome; NGS: Next generation sequencing; PDGFR: Platelet derived growth factor receptor; PDGFRB: Platelet derived growth factor receptor gene B; PDGFRβ: Platelet derived growth factor receptor beta; TKI: Tyrosine kinase inhibitor; VAC: Vincristine/actinomycine D/cyclophosphamide; VBL: Vinblastine

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Availability of data and materials

The datasets and/or the analyzed current case report are available from the corresponding author upon reasonable request.

Authors' contributions

PM performed the review of the literature and wrote the draft of the manuscript. OS and EM performed the DNA analysis of the *PDGRFB* gene. JN and RV designed and performed the phosphoproteomic analysis. JSo proposed to perform the NGS analysis and participated as clinical geneticist. KM took care of the patient and participated in the writing of the manuscript. OR took care of the patient and participated in the writing of the manuscript. MJ performed the histopathological analysis. AS performed the radiological evaluation and managed the MRI images. JSt proposed the study of molecular biology details of the case with a theranostic aim. All of the authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Written informed consent for the publication of their clinical details and/or clinical images was obtained from the parents of the patient. A copy of the consent form is available for review by the Editor of this journal.

Ethics approval and consent to participate

The study was approved by both the Ethics Committee of the University Hospital Brno on 9.6.2015 and the Ethics Committee of the School of Medicine Masaryk University on 23.6.2015, reference number 30/2015. All of the research described herein was conducted according to the Declaration of Helsinki. Written informed consent for the tissue and blood analysis and the *off-label* treatment of the child with the tyrosine kinase inhibitor was obtained from parents.

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Effects of Sunitinib and Other Kinase Inhibitors on Cells Harboring a *PDGFRB* Mutation Associated with Infantile Myofibromatosis

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Abstract: Infantile myofibromatosis represents one of the most common proliferative fibrous tumors of infancy and childhood. More effective treatment is needed for drug-resistant patients, and targeted therapy using specific protein kinase inhibitors could be a promising strategy. To date, several studies have confirmed a connection between the p.R561C mutation in gene encoding platelet-derived growth factor receptor beta (PDGFR-beta) and the development of infantile myofibromatosis. This study aimed to analyze the phosphorylation of important kinases in the NSTS-47 cell line derived from a tumor of a boy with infantile myofibromatosis who harbored the p.R561C mutation in PDGFR-beta. The second aim of this study was to investigate the effects of selected protein kinase inhibitors on cell signaling and the proliferative activity of NSTS-47 cells. We confirmed that this tumor cell line showed very high phosphorylation levels of PDGFR-beta, extracellular signal-regulated kinases (ERK) 1/2 and several other protein kinases. We also observed that PDGFR-beta phosphorylation in tumor cells is reduced by the receptor tyrosine kinase inhibitor sunitinib. In contrast, MAPK/ERK kinases (MEK) 1/2 and ERK1/2 kinases remained constitutively phosphorylated after treatment with sunitinib and other relevant protein kinase inhibitors. Our study showed that sunitinib is a very promising agent that affects the proliferation of tumor cells with a p.R561C mutation in PDGFR-beta.

Keywords: infantile myofibromatosis; receptor tyrosine kinases; platelet-derived growth factor receptor; protein kinase inhibitors; sunitinib; erlotinib; FR180204; U0126; targeted therapy

1. Introduction

Infantile myofibromatosis (IM; [MIM#228550]) is a disorder of mesenchymal proliferation characterized by the development of nonmetastatic tumors [1] that present as firm, flesh-colored to purple nodules usually located in the skin, subcutaneous tissues, bone, muscle or visceral organs [2,3]. This disease was described under different names, the name "infantile myofibromatosis" was first used in 1981 [4]. Although rare, with an incidence of 1 in 400,000 children, IM represents the most common proliferative fibrous tumor of infancy [5,6]. Myofibromas are usually present at birth or

develop shortly thereafter, and almost 90% of the tumors are diagnosed before the age of two years, with a median age of three months [6–8]. A male predominance has been reported, and the ratio of male to female patients varies from 1.5:1 to 1.8:1 [5].

IM clinically presents in three main forms: (1) Solitary, (2) multicentric without visceral involvement, and (3) multicentric with visceral involvement [6]. The prognosis is excellent in solitary or multicentric nonvisceral forms with a possibility of spontaneous regression of the lesions but is poor when detected in the viscera [9]. Surgical excision of a single lesion is the standard of care [8]. Multiple lesions or surgically unresectable lesions are treated using various therapeutics, such as anti-inflammatory drugs, interferon-alpha, vinblastine, vincristine, dactinomycin, cyclophosphamide and methotrexate [6,8].

The molecular pathogenesis of IM is not completely understood. Familial forms exhibiting autosomal dominant and recessive transmission have been reported over the past two decades [10]. In 2013, several point mutations in the platelet-derived growth factor receptor beta (PDGFR-beta) gene (*PDGFRB*) were identified to be associated with familial IM. A study of nine unrelated families diagnosed with IM revealed two disease-causing mutations in *PDGFRB*: c.1978C>A (p.P660T) and c.1681C>T (p.R561C) [1]. Interestingly, one family did not have either of these *PDGFRB* mutations, but all affected individuals had a c.4556T>C (p.L1519P) mutation in *NOTCH3*. The germline mutation c.1681C>T (p.R561C) in *PDGFRB* was also detected in 11 individuals with familial IM [7]. In addition, one individual harbored a c.1998C>A (p.N666K) somatic mutation. Very recently, a novel *PDGFRB* mutation (c.1679C>T; p.P560L) was identified in a 3-generation family with multicentric IM [11].

Platelet-derived growth factors (PDGFs) and PDGF receptors (PDGFRs) have important functions in the regulation of cell growth and survival [12]. The PDGF family consists of four structurally related single polypeptide units that constitute five functional homo- or heterodimers: PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD [13]. PDGFs act via two receptor tyrosine kinases (RTKs), PDGFR-alpha and PDGFR-beta [14]. Both receptors can activate many major signal transduction pathways, including the Ras/MAPK, PI3K/Akt and phospholipase C-gamma pathways [15].

Moreover, other genes were associated with IM etiology, which demonstrates the possible genetic heterogeneity of this disease. As mentioned above, a connection between a c.4556T>C (p.L1519P) mutation in *NOTCH3* and IM was described in one study [1]. Human cells express four different Notch receptors, Notch 1–4, each encoded by a different gene [16]. The expression of *PDGFRB* can be regulated by Notch activity, as PDGFR-beta expression can be robustly upregulated by Notch 1 and Notch 3 signaling [17]. Another example is a c.511G>C (p.V171L) mutation in the potential tumor suppressor *NDRG4* that was associated with IM in one case [18]. In the same year, it was demonstrated that the c.1276G>A (p.V426M) mutation in *PTPRG* (protein tyrosine phosphatase, receptor type G) was able to substantially influence the penetrance of a c.1681C>T (p.R561C) mutation in *PDGFRB* [19]. *PTPRG* encodes an enzyme that could dephosphorylate PDGFR-beta and thus reduce PDGFR-beta activity [19,20].

A recent work revealed that two IM-associated mutations in *PDGFRB*, c.1681C>T (p.R561C) and c.1998C>A (p.N666K), constitutively activate PDGFR-beta and can induce cancer development in vivo [21]. The same study showed that cells harboring p.R561C and p.N666K mutations are sensitive to specific tyrosine kinase inhibitors, which were able to decrease PDGFR-beta phosphorylation and downstream signaling. These results suggested that blocking PDGFR-beta activity would offer a therapeutic option for IM treatment. Indeed, in a recently published study, targeted treatment with sunitinib and low-dose vinblastine led to a robust response in a child with refractory multiple IM and a c.1681C>T (p.R561C) mutation in *PDGFRB* [8].

In this work, we demonstrate for the first time the efficacy of sunitinib, erlotinib, U0126 and FR180204 on the cell line harboring a c.1681C>T (p.R561C) *PDGFRB* mutation found in patients with IM. Sunitinib is known as an inhibitor of several kinases, including PDGFR-beta [22], erlotinib is an inhibitor of epidermal growth factor receptor (EGFR) [23], U0126 inhibits MEK1/2 phosphorylation [24], and FR180204 inhibits ERK1/2 phosphorylation. These inhibitors were chosen

on the basis of our previous findings [8] as well as on the results of subsequent phosphoprotein profiling of the NSTS-47 cell line.

2. Results

2.1. Germline Mutations in PDGFRB Were Identified in Both Children, and the Same Mutation in PDGFRB Was Confirmed in NSTS-47 Cells

Genetic analyses revealed that both siblings harbor a heterozygous germline c.1681C>T (p.R561C) mutation in the *PDGFRB* gene (Table 1). It was also confirmed that NSTS-47 cell line harbors the same heterozygous germline mutation c.1681C>T (p.R561C) in *PDGFRB*.

Table 1. Germline mutations identified in patients.

Gender	Age	*PDGFRB* Mutation
Male	3.5 months	c.1681C>T (p.R561C)
Female	8 years	c.1681C>T (p.R561C)

2.2. PDGFR-Beta, EGFR and ERK1/2 Kinases Are Highly Phosphorylated in Cells Harboring c.1681C>T (p.R561C) Mutation in PDGFRB

Given that both siblings and NSTS-47 cells harbor the c.1681C>T (p.R561C) mutation in *PDGFRB* and that PDGFR-beta c.1681C>T (p.R561C) mutants are constitutively phosphorylated and can activate various signaling pathways [21], we assessed the phosphorylation level of 49 RTKs and 26 other signaling proteins in tumor samples as well as in NSTS-47 cells. NSTS-47 cells were harvested, and phosphorylation levels were analyzed after cultivation for 24 h in Dulbecco's modified Eagle's medium (DMEM) without fetal calf serum (FCS) to eliminate the effects of various serum growth factors on the phosphorylation of the studied proteins. The screening of all 75 proteins showed that PDGFR-beta, EGFR (Figure 1) and ERK1/2 (Figure 2) kinases exhibited very high levels of phosphorylation in all samples. High levels of phosphorylation were also observed for ROR2, AXL (Figure 1), HSP27 and p38-gamma (Figure 2). These results confirmed that some kinases (namely, PDGFR-beta, EGFR and ERK1/2) were constitutively activated, as the high phosphorylation levels of these proteins were easily detectable in both tumor samples and in NSTS-47 cells after cultivation under serum-free conditions for 24 h.

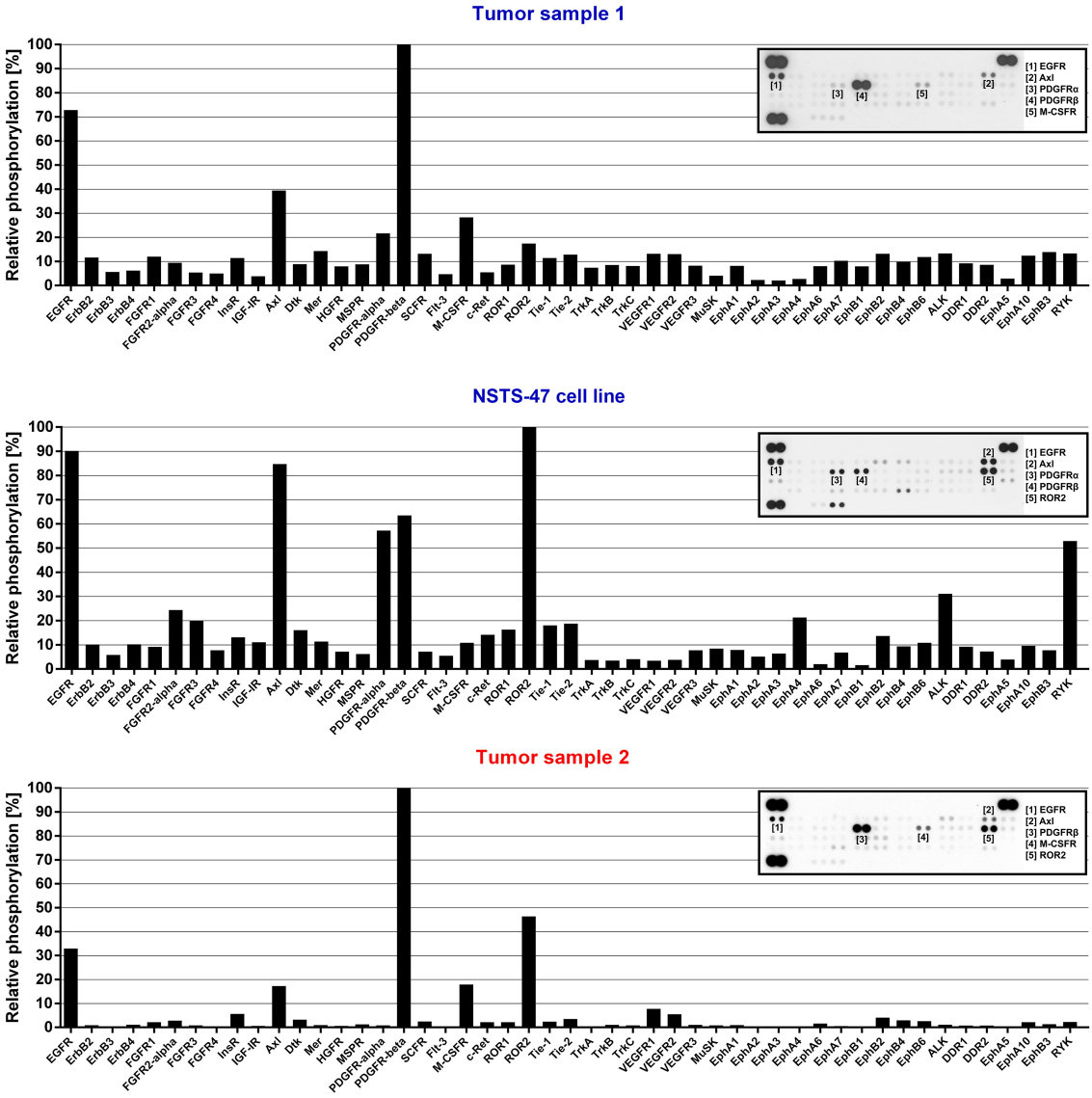

Figure 1. Phospho-receptor tyrosine kinases (RTK) array analysis. The relative phosphorylation of 49 RTKs was analyzed in tumor tissue obtained from the boy when he was 3.5 months old (Tumor sample 1), in the NSTS-47 cell line (derived from a tumor tissue of the boy obtained when he was 1 year and 7 months old) and in the tumor tissue of his 8-year-old sister (Tumor sample 2). platelet-derived growth factor receptor beta (PDGFR-beta) and epidermal growth factor receptor (EGFR) exhibited high levels of phosphorylation in all cases. Phosphorylation in NSTS-47 cells was measured after 24 h of serum-free cultivation. The array images captured using X-ray film are shown for each sample, and the five most phosphorylated receptor tyrosine kinases (RTKs) are marked. The upper part of the figure (Tumor sample 1) was already published in our previous case report [8] under the Creative Commons Attribution 4.0 International License.

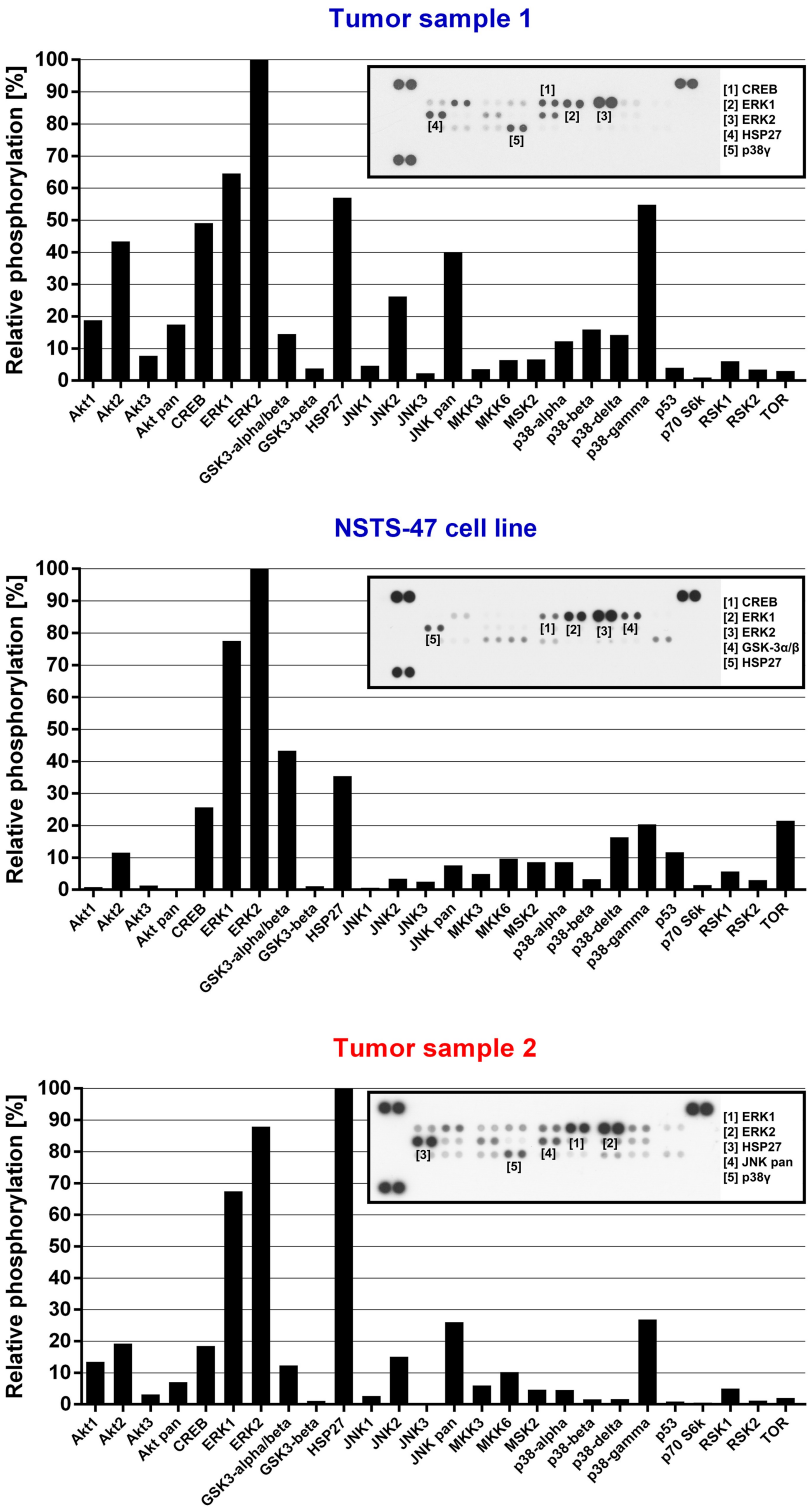

Figure 2. Phospho-mitogen-activated protein kinase (MAPK) array analysis. The relative phosphorylation of 26 signaling proteins, including 9 MAPKs, was detected in tumor tissue obtained from the boy when he was 3.5 months old (Tumor sample 1), in the NSTS-47 cell line (derived from a tumor tissue of the boy obtained when he was 1 year and 7 months old) and in the tumor tissue of his 8-year-old sister (Tumor sample 2). ERK1/2 exhibited high levels of phosphorylation in all cases. Phosphorylation levels in NSTS-47 cells was measured after 24 h of serum-free cultivation. The array images captured using X-ray film are shown for each sample, and the five most phosphorylated proteins are marked.

2.3. NSTS-47 Cells Are Sensitive to Sunitinib and Erlotinib

It was confirmed that cells with the mutation c.1681C>T (p.R561C) in *PDGFRB* are sensitive to the tyrosine kinase inhibitors imatinib, nilotinib and ponatinib [21]. Given the phosphorylation profile in the NSTS-47 cell line, whether specific tyrosine kinase inhibitors could affect the proliferation of this cell line was assessed. NSTS-47 cells were first treated with sunitinib. Sunitinib was chosen for several reasons: (1) The NSTS-47 cell line harbors a c.1681C>T (p.R561C) mutation in *PDGFRB*, and PDGFR-beta was substantially phosphorylated in these cells; (2) sunitinib treatment inhibits PDGFR-beta phosphorylation [25]; and (3) sunitinib was successfully used to treat the boy with IM whose tumor tissue was used to generate the NSTS-47 cell line [8].

Cells were treated for six days with various concentrations of sunitinib, and after incubation, the proliferative activity was determined using the MTT assay. At sunitinib concentrations of 50 and 100 nM, which can be achieved in the plasma of children treated with sunitinib [26], the proliferative activity of NSTS-47 cells was significantly decreased (Figure 3A). In addition, 50 nM and 100 nM sunitinib decreased the proliferative activity of NSTS-47 cells to 75% and 73%, respectively, after six days.

To verify whether the observed effect of sunitinib is robust, NSTS-47 cells were cultivated with sunitinib in medium supplemented with PDGF-BB. A significant decrease in proliferative activity was observed after sunitinib treatment even when the cells grew in medium supplemented with PDGF-BB at a high concentration of 10 ng/mL (Figure 3B). In some experiments, the cultivation medium was changed every 24 h, and new medium with fresh inhibitor and fresh PDGF-BB was added (at medium changes) to prevent the potential degradation of sunitinib and PDGF-BB (Figure 3C).

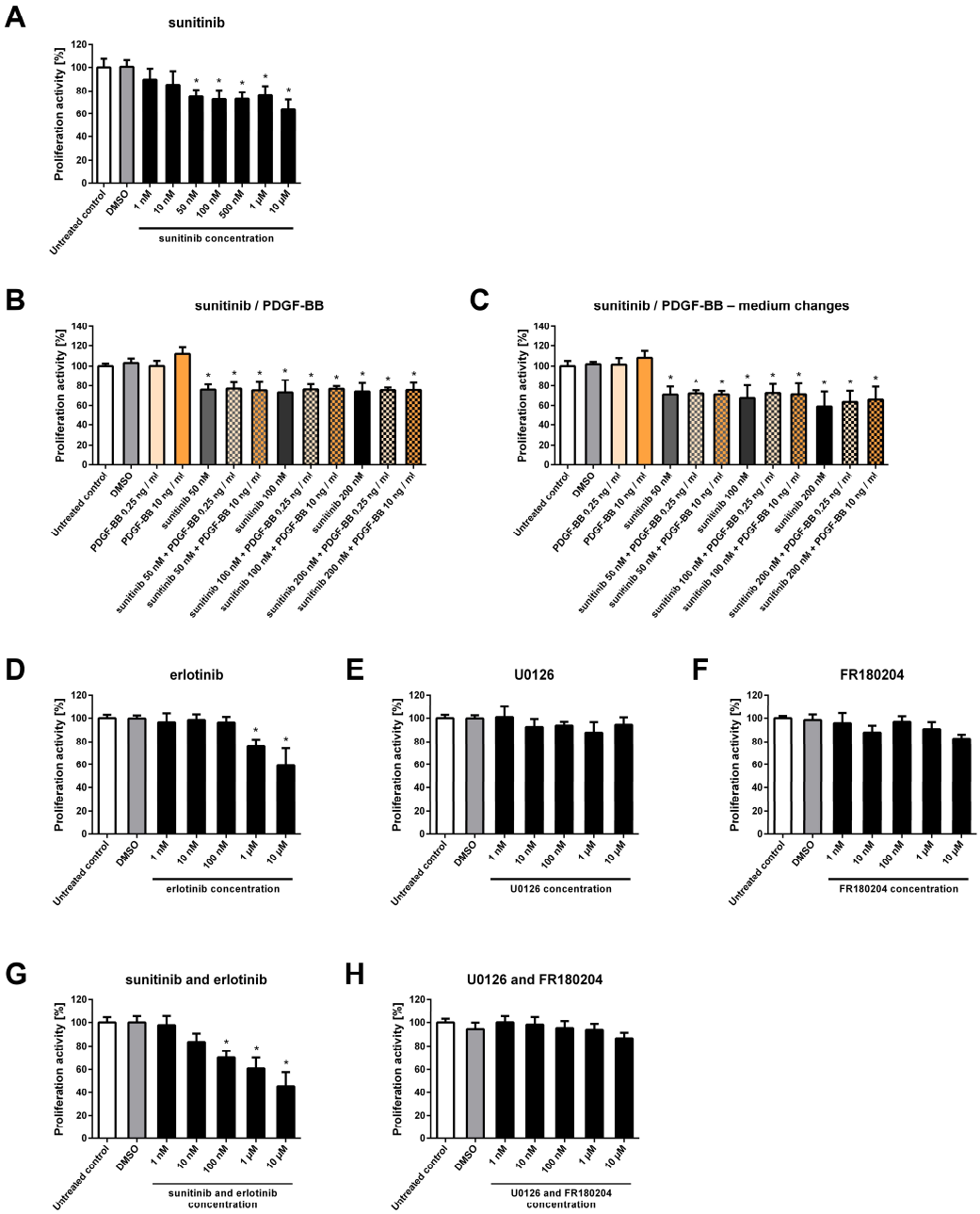

Figure 3. Proliferative activity of NSTS-47 cells after various experimental treatments. Proliferative activity was measured using an MTT assay after 6 days of incubation. The data represent the mean ± SD. Experiments were repeated three times in hexaplicate (**A**,**D**–**H**) or in triplicate (**B**,**C**). * $p < 0.05$ indicates a significant difference compared to control cells. (**A**) Sunitinib significantly decreased the proliferative activity of NSTS-47 cells. (**B**) NSTS-47 cells were sensitive to sunitinib, and this effect was not influenced by the presence of PDGF-BB at a high concentration (10 ng/mL). (**C**) Medium containing inhibitor and PDGF-BB was changed every 24 h during cultivation, which had no significant effect on the efficacy of the inhibitor. (**D**) NSTS-47 cells were also sensitive to erlotinib, as this inhibitor significantly affected cell proliferation. (**E**) No significant effect was observed after U0126 treatment. (**F**) FR180204 also did not significantly affect proliferative activity. (**G**) The combination of erlotinib and sunitinib significantly decreased the proliferative activity of NSTS-47 cells. (**H**) The combination of U0126 and FR180204 did not have a significant effect on NSTS-47 cell proliferation.

Next, NSTS-47 cells were treated with erlotinib, U0126 and FR180204. These three inhibitors were chosen based on EGFR and ERK1/2 phosphorylation in NSTS-47 cells (Figures 1 and 2). The ability of the combination of sunitinib and erlotinib to block both highly phosphorylated RTKs was tested, and a combination of U0126 and FR180204 was used to block the MEK/ERK signaling pathway.

At an erlotinib concentration of 1 μM, which can be achieved in the plasma of children treated with erlotinib [27], the proliferative activity of the NSTS-47 cell line was significantly decreased to 75% after 6 days of cultivation (Figure 3D). In contrast, NSTS-47 cells were not sensitive to U0126 and FR180204 because treatment of the NSTS-47 cell line with these inhibitors did not induce a significant decrease in proliferative activity (Figure 3E,F).

The combination of erlotinib and sunitinib also significantly decreased the proliferative activity of NSTS-47 cells (Figure 3G), but the effect of this combined treatment was similar to the effects of sunitinib or erlotinib alone. For instance, 100 nM sunitinib and 100 nM erlotinib decreased the proliferative activity to 70% (Figure 3G), but 100 nM sunitinib alone decreased the proliferative activity to 73% (Figure 3A). Another example is the combination of 1 μM erlotinib and 1 μM sunitinib; this treatment decreased the proliferative activity to 61% (Figure 3G), but 1 μM erlotinib alone decreased the proliferative activity to 75% (Figure 3D), and 1 μM sunitinib decreased the proliferative activity to 76% after six days (Figure 3A). Therefore, the combination of sunitinib and erlotinib did not have a significant additional effect on the reduction of NSTS-47 cell proliferation. In addition, the combination of U0126 and FR180204 did not show any significant effect on proliferative activity (Figure 3H).

Taken together, our results demonstrate that sunitinib and erlotinib can significantly decrease the proliferative activity of NSTS-47 cells, which harbor a c.1681C>T (p.R561C) mutation in *PDGFRB*, at concentrations that are achievable for these inhibitors in children plasma. However, the combination of sunitinib and erlotinib did not show an additional significant effect on cell proliferation. The inhibitors FR180204 and U0126 also did not have a significant effect on NSTS-47 cell proliferation.

2.4. PDGFR-Beta and EGFR Exhibited Ligand-Dependent Tyrosine Phosphorylation

Considering that only some kinase inhibitors significantly decreased the proliferative activity of the NSTS-47 cell line, detailed analyses of target kinases that should be affected by previously used inhibitors were performed using Western blotting. First, it was observed that the constitutively phosphorylated receptors PDGFR-beta and EGFR in NSTS-47 cells can respond to their ligands: Our results show that phosphorylation of both receptors was considerably increased in response to PDGF-BB or EGF (Figure 4A,B). Cell populations were serum starved for 24 h and then stimulated for 15, 30 or 60 min using two different concentrations of PDGF-BB or EGF. The cells that were serum starved for only 24 h and cells that were cultivated with FCS were used as negative controls. Receptor phosphorylation was significantly increased after 15 min, and then decreased in a time-dependent manner. Surprisingly, serum-starved cells that were not stimulated with PDGF-BB or EGF also exhibited an increase in receptor phosphorylation, in comparison to serum-cultivated cells. These experiments demonstrated that both receptors were functional and were able to activate downstream signaling molecules.

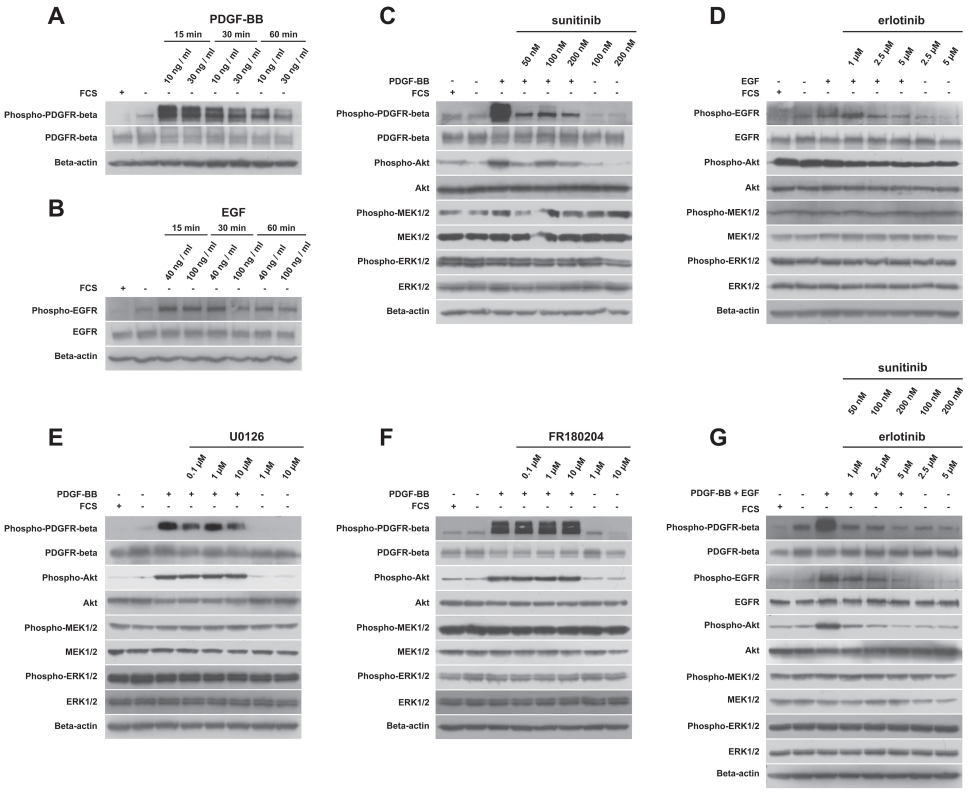

Figure 4. Analysis of protein phosphorylation. (**A**) PDGFR-beta phosphorylation is increased in response to PDGF-BB. Cells were stimulated for 15, 30 or 60 min using two different concentrations (10 ng/mL and 30 ng/mL) of PDGF-BB. (**B**) EGFR phosphorylation is increased in response to epidermal growth factor (EGF). Cells were stimulated for 15, 30 or 60 min using two different concentrations (40 ng/mL and 100 ng/mL) of EGF. (**C**) Sunitinib was able to decrease PDGFR-beta and Akt phosphorylation but not MEK1/2 and ERK1/2 phosphorylation. (**D**) Erlotinib decreased EGFR and Akt phosphorylation but had no effect on MEK1/2 and ERK1/2 phosphorylation. (**E**) U0126 treatment did not decrease MEK1/2 phosphorylation. (**F**) FR180204 treatment did not cause any changes in ERK1/2 phosphorylation. (**G**) The combination of sunitinib and erlotinib decreased PDGFR-beta, EGFR and Akt phosphorylation, but MEK1/2 and ERK1/2 phosphorylation was not affected.

2.5. Detailed Analysis of Signaling Pathways Revealed Constitutive Phosphorylation of MEK1/2 and ERK1/2 Proteins

In the next step, we analyzed the phosphorylation of PDGFR-beta, EGFR and downstream kinases, which can be activated by these RTKs after treatment with kinase inhibitors. In all experiments, cells were cultivated for 24 h in medium containing an inhibitor but not FCS. After 24 h, some cells were stimulated with PDGF-BB or/and EGF for 15 min to observe the effects of inhibitors on ligand-stimulated cells. Cells that were serum starved for only 24 h, and cells that were cultivated with FCS were used as negative controls.

Sunitinib alone decreased the phosphorylation of PDGFR-beta (Figure 4C). Akt phosphorylation was also decreased after sunitinib treatment, but a substantial decrease in MEK1/2 and ERK1/2 phosphorylation was not observed. Erlotinib decreased the phosphorylation of EGFR, but only at higher concentrations, and Akt phosphorylation was also slightly decreased (Figure 4D). No effect of erlotinib on MEK1/2 and ERK1/2 phosphorylation was observed. Surprisingly, U0126 did not decrease the phosphorylation of MEK1/2 (Figure 4E). Similarly, FR180204 treatment had no effect on ERK1/2 phosphorylation (Figure 4F). As expected, the combination of sunitinib and erlotinib markedly decreased the phosphorylation of PDGFR-beta, EGFR and Akt, but no effect was observed on MEK1/2 and ERK1/2 phosphorylation (Figure 4G).

Altogether, sunitinib and erlotinib showed inhibitory effects on RTKs and Akt. Interestingly, no substantial changes in MEK1/2 and ERK1/2 phosphorylation were observed after treatment with any inhibitor.

2.6. Serum Starvation of NSTS-47 Cells Induces an Increase in PDGFA Expression

In some cases, our data indicated higher phosphorylation of PDGFR-beta and EGFR in serum-starved cells than in cells cultivated in DMEM supplemented with FCS (Figure 4A,B). Therefore, the expression of selected EGFR and PDGFR-beta ligands was measured to investigate whether there is a possible autocrine PDGF/PDGFR or EGF (TGF-alpha)/EGFR signaling loop that could contribute to the higher phosphorylation of RTKs. Expression of *EGF*, *PDGFA*, *PDGFB* and *TGFA* was analyzed under normal serum conditions (DMEM supplemented with 20% FCS) and under serum starvation conditions using qPCR. Substantial differences were observed in the transcriptional response of serum-starved cells (Figure 5). qPCR analyses also showed increased levels of *PDGFA* expression, while *EGF* and *PDGFB* mRNA levels were not significantly influenced by serum starvation, and *TGFA* expression was considerably decreased.

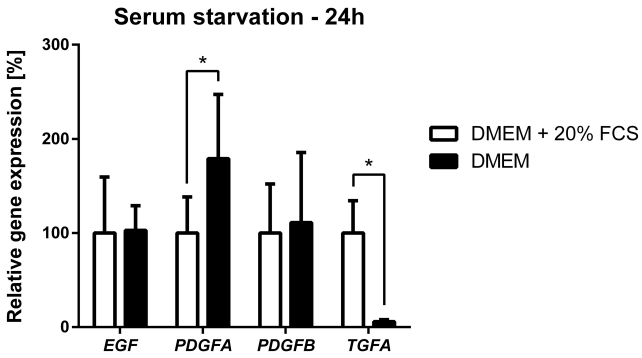

Figure 5. Effect of serum starvation on *EGF*, *PDGFA*, *PDGFB* and *TGFA* expression in the NSTS-47 cell line. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FCS or in DMEM without FCS. After 24 h, cells were harvested, and the expression of selected genes was analyzed using qPCR. The results represent the mean ± SD of nine (six in case of *PDGFB*) independent experiments. * $p < 0.05$ indicates statistically significant differences.

3. Discussion

IM is a rare disorder of mesenchymal proliferation that is characterized by the development of nonmetastatic tumors [1]. Several studies have confirmed that specific point mutations in the *PDGFRB* gene are involved in the pathogenesis of IM [1,7,11]. However, mutations in the *PDGFRB* gene presumably show incomplete penetrance and variable expressivity, and other genes may be involved in the pathogenesis of IM [1,18,19].

The main goal of this study was to analyze the effects of various protein kinase inhibitors (PKIs) on the NSTS-47 cell line, which harbors the IM-associated c.1681C>T (p.R561C) mutation in *PDGFRB*. The results showed that sunitinib, a potent inhibitor of PDGFR-beta phosphorylation, can significantly decrease the proliferation of NSTS-47 cells.

Previously published results [21] show that PDGFR-beta p.R561C mutant cells have constitutively phosphorylated PDGFR-beta and are able to induce the phosphorylation of ERK1/2, PLC-gamma, STAT3, STAT5 and Akt in the absence of PDGF. These results are in accordance with our observations. We found that PDGFR-beta and ERK1/2 kinases were highly phosphorylated in both s and even in NSTS-47 cells that were serum starved for 24 h. We also detected increased phosphorylation of Akt2 in Tumor Sample 1.

The same study that revealed a role for the p.R561C mutation in PDGFR-beta [21] showed that imatinib, nilotinib, and ponatinib can decrease PDGFR-beta phosphorylation and inhibit cell

proliferation. We studied the effects of sunitinib, a multi-tyrosine kinase inhibitor that is able to target PDGFR-beta. Sunitinib was chosen because siblings from whom tumor tissue samples were obtained responded very well to treatment with this inhibitor [8]. Sunitinib alone significantly decreased the proliferative activity of the NSTS-47 cell line, and this finding could explain the response of the siblings to the targeted therapy.

Western blot analyses showed that sunitinib is able to decrease the phosphorylation of mutant PDGFR-beta even in the presence of high PDGF-BB levels and can also decrease the phosphorylation of Akt. Because activated Akt is a well-established survival factor [28], these effects of sunitinib on PDGFR-beta and Akt phosphorylation can explain why sunitinib reduced the proliferative activity of NSTS-47 cells.

A similar inhibitory effect was observed for EGFR and erlotinib (the inhibitor of EGFR phosphorylation). Erlotinib also decreased NSTS-47 cell proliferation, and Western blot analysis showed that it was able to decrease EGFR and Akt phosphorylation. However, neither sunitinib nor erlotinib inhibited the phosphorylation of the corresponding receptor completely, and some receptor molecules remained phosphorylated even when high doses of those inhibitors were used.

Surprisingly, phosphorylation of MEK1/2 and ERK1/2 proteins was not significantly influenced by any inhibitor. This observation could explain why sunitinib and erlotinib incompletely decreased proliferative activity and why U0126 and FR180204 did not influence proliferative activity. MEK1/2 and ERK1/2 belong to the Ras/MAPK signaling cascade, which transmits signals from receptors and participate in regulating the cell cycle, apoptosis and differentiation [29]. All tyrosine kinase inhibitors have been previously shown to be able to simultaneously decrease PDGFR-beta and ERK1/2 phosphorylation, which resulted in the inhibition of proliferative activity [21]. In NSTS-47 cells, sunitinib and erlotinib decreased the phosphorylation of PDGFR-beta, EGFR and Akt, but for yet unknown reasons, MEK1/2 and ERK1/2 kinases remained phosphorylated at levels that were comparable with those detected in untreated cells.

Interestingly, incomplete penetrance of the c.1681C>T (p.R561C) mutation was found in a family with two children suffering from IM [19]. Genetic analyses revealed a c.1681C>T (p.R561C) mutation in *PDGFRB* in both siblings and, surprisingly, also in their healthy mother. However, both siblings had inherited a heterozygous c.1276G>A (p.V426M) mutation in *PTPRG* from their healthy father. The *PTPRG* gene encodes a protein called receptor-type tyrosine-protein phosphatase gamma that can dephosphorylate PDGFR-beta [19,20]. Therefore, the mutation in *PTPRG* could probably decrease the efficiency of the phosphatase to dephosphorylate its substrates and thus positively influence the phosphorylation of PDGFR-beta and the penetrance of mutant *PDGFRB* [19].

Finally, our analyses of gene expression showed that the phosphorylation status of PDGFRs in NSTS-47 cells was not influenced by only mutations in PDGFR-beta. We analyzed the gene expression levels of *EGF*, *PDGFA*, *PDGFB* and *TGFA* in NSTS-47 cells that were serum starved for 24 h. The expression of *TGFA* decreased, but no difference was observed in the expression of *EGF* and *PDGFB*; however, *PDGFA* gene expression was significantly increased. The increase in *PDGFA* expression was unexpected and could result in the stimulation of cells via an autocrine mechanism, an increase in PDGFR-alpha phosphorylation and improved survival of NSTS-47 cells in the absence of serum.

4. Materials and Methods

4.1. Tumor Samples

Two tumor samples and one tumor-derived cell line were used in this study. Tumor Sample 1 was obtained from a 3.5-month-old infant boy suffering from inborn generalized IM, and Tumor Sample 2 was obtained from his 8-year-old sister who was suffering from a skull base tumor and had a history of spontaneous regression of subcutaneous lesions. The Research Ethics Committee of the School of Medicine (Masaryk University, Brno, Czech Republic) approved the study protocol, and written informed consent was obtained from legal guardians of the siblings. A case report concerning these siblings was published recently [8].

4.2. Cell Line and Cell Culture

The NSTS-47 cell line was established in our laboratory with the procedure previously described [30]. A tumor sample was obtained from the same boy mentioned in the previous paragraph during curative surgical procedure when he was 1 year and 7 months old. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% fetal calf serum (FCS), 2 mM glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin (all purchased from GE Healthcare Europe GmbH, Freiburg, Germany). The cell line was maintained under standard conditions at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultured one or two times per week. Cells from passage number 8 to 19 were used for experiments.

4.3. Genetic Analyses

The mutation in *PDGFRB* was identified by Sanger sequencing using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and confirmed by whole exome sequencing (WES). In all cases, WES was performed using the TruSeq Exome Kit, NextSeq® 500/550 Mid Output Kit v2 and NextSeq 500 (all Illumina, San Diego, CA, USA).

4.4. Chemicals

Sunitinib, erlotinib, U0126 (all purchased from Cell Signaling Technology, Danvers, MA, USA) and FR180204 (Sigma-Aldrich, St. Louis, MO, USA) were prepared as a 20 mM stock solution in dimethyl sulfoxide (DMSO) and stored at −20 °C. PDGF-BB (Cell Signaling Technology) was prepared at a concentration of 100 μg/mL in 20 mM citric acid (pH 3.0) supplemented with 0.8% BSA (bovine serum albumin) and stored at 4 °C. EGF (Sigma-Aldrich) was prepared at a concentration of 100 μg/mL in 10 mM HCl and stored at 4 °C. For the determination of proliferative activity, concentrations of protein kinase inhibitors (PKIs) ranging from 0.001 to 10 μM and PDGF-BB concentrations of 0.25 and 10 ng/mL were tested. For Western blot analyses, PKI concentrations ranging from 0.05 to 10 μM, PDGF-BB concentrations of 10 and 30 ng/mL and EGF concentrations of 40 and 100 ng/mL were used.

4.5. Phospho-RTK and Phospho-MAPK Array Analysis

The relative phosphorylation levels of 49 RTKs were analyzed using the Human Phospho-RTK Array kit (R&D Systems, Minneapolis, MN, USA), and the relative phosphorylation levels of 26 proteins, including 9 MAPKs, were determined using the Human Phospho-MAPK Array kit (R&D Systems) according to the manufacturer's protocol. The levels of phosphorylation were quantified using ImageJ software [31] and normalized to control spots and the background. The analysis was performed as described in previous studies [8,32].

4.6. MTT Assay

The MTT assay was used to determine the proliferative activity of the NSTS-47 cell line. A total of 10^3 cells were seeded in 200 μL of culture medium into each well of 96-well microplates, and cells were allowed to adhere overnight. The next day, the medium was carefully removed, and fresh medium containing various concentrations of chemicals described above or control medium was added. The microplates were incubated under standard conditions. To evaluate changes in cell proliferation, the medium was removed and replaced with 200 μL of fresh DMEM containing 3-(4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a concentration of 0.5 mg per mL. The microplates were then incubated at 37 °C for 3.5 h. The medium was carefully removed, and the formazan crystals were dissolved in 200 μL of DMSO. The absorbance was measured at 570 nm using a Sunrise Absorbance Reader (Tecan, Männedorf, Switzerland), with a reference absorbance at 620 nm.

4.7. Western Blotting and Immunodetection

Whole-cell extracts were loaded onto 10% polyacrylamide gels, electrophoresed, and blotted on polyvinylidene difluoride membranes (Bio-Rad Laboratories, Munich, Germany). The membranes were blocked with 5% nonfat dry milk in phosphate buffered saline (PBS) containing 0.1% Tween-20 and incubated overnight with the corresponding primary antibody. The primary and secondary antibodies used in this study are shown in Table 2. Membranes were incubated with corresponding secondary antibodies for 1 h. ECL-Plus detection was performed according to the manufacturer's instructions (GE Healthcare, Little Chalfont, UK).

Table 2. Primary and secondary antibodies.

Primary Antibodies			
Antigen	Manufacturer	Catalog No.	Dilution
Beta-actin	Sigma-Aldrich	A5441	1:20,000
Akt (pan)	Cell Signaling Technology	4691	1:1000
Phospho-Akt (Ser473)	Cell Signaling Technology	4060	1:2000
ERK1/2	Cell Signaling Technology	4695	1:1000
Phospho-ERK1/2 (Thr202/Tyr204)	Cell Signaling Technology	4370	1:2000
MEK1/2	Cell Signaling Technology	9122	1:1000
Phospho-MEK1/2 (Ser217/221)	Cell Signaling Technology	9121	1:1000
EGFR	Cell Signaling Technology	2646	1:1000
Phospho-EGFR (Tyr1068)	Cell Signaling Technology	2236	1:1000
PDGFR-beta	Cell Signaling Technology	3169	1:1000
Phospho-PDGFR-beta (Tyr751)	Cell Signaling Technology	4549	1:1000

Secondary antibodies				
Specificity	Conjugate	Manufacturer	Catalog No.	Dilution
Anti-Mouse IgG	horseradish peroxidase	Cell Signaling Technology	7076	1:2000–1:20,000
Anti-Rabbit IgG	horseradish peroxidase	Cell Signaling Technology	7074	1:2000

4.8. RT-qPCR

The relative expression levels of selected genes were studied using RT-qPCR. Total RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich), and RNA concentration and purity were determined spectrophotometrically. For all samples, equal amounts of RNA were reverse transcribed into cDNA using M-MLV reverse transcriptase (Top-Bio, Prague, Czech Republic). RT-qPCR was carried out in 10 µL reaction volumes using the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) and analyzed using the 7500 Fast Real-Time PCR System and 7500 Software v. 2.0.6 (both Life Technologies, Carlsbad, CA, USA). Changes in the transcript levels were determined using the $2^{-\Delta\Delta CT}$ method [33]. The housekeeping gene *HSP90AB1* was used as an endogenous reference control. The primers used in this study are listed in Table 3.

Table 3. Primers.

Gene	Gene Symbol	Primer Sequence
Epidermal growth factor	*EGF*	F: 5′-AGGATTGACACAGAAGGAACCAA-3′ R: 5′-ACATACTCTCTCTTGCCTTGACC-3′
Heat shock protein 90 alpha family class B member 1	*HSP90AB1*	F: 5′-CGCATGAAGGAGACACAGAA-3′ R: 5′-TCCCATCAAATTCCTTGAGC-3′
Platelet derived growth factor subunit A	*PDGFA*	F: 5′-TCCGTAGGGAGTGAGGATTCTTT-3′ R: 5′-GGCTTCTTCCTGACGTATTCCA-3′
Platelet derived growth factor subunit B	*PDGFB*	F: 5′-GATCCGCTCCTTTGATGATCTCC-3′ R: 5′-ATCTCGATCTTTCTCACCTGGAC-3′
Transforming growth factor alpha	*TGFA*	F: 5′-TGCCACTCAGAAACAGTGGTC-3′ R: 5′-AGTCCGTCTCTTTGCAGTTCTT-3′

F, forward primer; R, reverse primer.

4.9. Statistical Analysis

Quantitative data are shown as the mean ± standard deviation (SD). Data from MTT assays were analyzed using one-way ANOVA followed by Dunnett's test; $p < 0.05$ was considered statistically significant. The qPCR data were analyzed using the Mann-Whitney test (two-tailed); $p < 0.05$ was considered statistically significant.

5. Conclusions

To conclude, our work demonstrated that tumor cells with the c.1681C>T (p.R561C) mutation in *PDGFRB* show high levels of PDGFR-beta and ERK1/2 phosphorylation. Furthermore, our data support the use of specific tyrosine kinase inhibitors targeting PDGFR-beta phosphorylation as a treatment suitable for IM. This is the first study to show that sunitinib is able to reduce the proliferative activity of IM cells with a c.1681C>T (p.R561C) mutation in vitro.

Author Contributions: J.N., R.V. and J.S. designed the study. J.S., P.M. (Peter Mudry) and K.P. provided tumor samples and relevant clinical data. H.N. and O.S. performed genetic analyses. M.S., P.M. (Petra Macigova) and J.N. designed and performed experiments with NSTS-47 cell line. M.S. and R.V. composed the manuscript. All authors reviewed and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
IM	infantile myofibromatosis
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MTT	3-(4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PDGFR	platelet-derived growth factor receptor
PKIs	protein kinase inhibitors
RTKs	receptor tyrosine kinases
TGFA	transforming growth factor alpha
WES	whole exome sequencing

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Letter to Editor: F.S. Kaplan, et al., Early clinical observations on the use of imatinibmesylate in FOP: A report of seven cases, Bone (2017)

We read with great interest the article published by F.S. Kaplan et al. Early clinical observations on the use of imatinibmesylate in FOP: A report of seven cases, Bone (2017). Following this [1] we would like to present a case report of one our patient with FOP successfully responding to sunitinib.

The twenty months old girl was admitted with soft tissue swelling of back part of the neck, right axilla and jugulum. Lesions were reddish, warm and some of them melted away, however causing limited mobility of the neck and upper limbs. First local histopathology revealed infantile (lipo)fibromatosis with low mitotic activity. Staging showed multiple lesions of the neck, chest and back and the patient was referred to us. Rebiopsy of lesions in order to provide fresh tissue to target the treatment of suspected soft tissue neoplasm showed similar histopathology findings, with negativity of both NOTCH and PDGFRB gene mutations. The patient started therapy for infantile myofibromatosis with low dose vinblastine and methotrexate. After four weeks of treatment we observed clinical progression with rapid appearance (within hours) of new lesions, compatible with flair-up behavior and fever. One dose of methylprednisolon resulted into partial regression of flair-ups but no efficacy to tumors was observed. Phospoproteomics showed high levels of PDGFRβ phosphorylation [2] transcriptome showed overexpression of NTRK 2 and 3, however no NTRK gene fusions were found. After discussion with parents combination of low dose of vinblastine plus anti PDGFRβ drug sunitinib in initial dose 0.5 mg/kg/day plus celecoxibe as a COX-2 inhibitor was started. After 1 month dose of sunitinib was escalated to 0.65 mg/kg/day. The whole exome sequencing revealed pathogenic R206H mutation in AVCR1 gene, which confirmed diagnosis of FOP. Sunitinib continued as 0.65 mg/kg/every other day. Active lesions slowly regressed and flair-ups almost completely ceased.

At the time of writing of this report, only rare and compared to pretreatment period very mild flair-ups with no progressive motion loss are observed. The patient is on treatment for almost two years. During the treatment we did not observe any other grade III and higher side effects except for one uneventful episode of febrile neutropenia.

This case report underlines challenges with ultra-orphan disease like FOP from diagnosis when such children are often referred to oncologist for off label treatment. Our patient is the youngest child treated with tyrosine-kinase inhibitor for management of FOP in the English literature. Inhibition of up-regulated PDGFRα, PDGFRβ, c-kit, HIF1alfa and other proinflammatory and proliferative pathways by sunitinib or imatinib seems worth to be considered in early phases of FOP having in mind nontrivial toxicity of experimental treatment with palovarotene [3] as well.

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Recurrent laryngeal papillomatosis: successful treatment with human papillomavirus vaccination

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Recurrent laryngeal papillomatosis: successful treatment with human papillomavirus vaccination

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ABSTRACT

The authors describe the case of a 5-year-old girl with recurrent laryngeal papillomatosis (RLP) due to human papillomavirus (HPV) type 11, who required frequent surgical treatment. Complete recovery occurred after HPV vaccination (Gardasil). Confirmed remission of RLP has continued during the 17 months of follow-up since vaccination. The authors conclude that HPV vaccination may represent a new therapeutic option in this situation.

CASE REPORT

The index case was born at term following an uncomplicated labour. Her mother had no clinical signs of human papillomavirus (HPV) infection. Psychomotor development was normal and the child had occasional mild upper respiratory tract infections. At the age of 2 years she gradually developed a hoarse voice and was referred to the local hospital. After ear-nose-throat (ENT) clinical examination and direct optical laryngoscopy, the patient was diagnosed with laryngeal papillomatosis and surgical laser ablation of papillomas was performed. Subsequent histological examination of tissue showed papillary structures covered by acanthotic squamous epithelium with mixed inflammatory cells in stroma. Final pathological diagnosis was squamous papilloma with mild dysplasia.

The patient was frequently seen by an ENT specialist during follow-up. Within 2 years of diagnosis there were six relapses with development of papillomas necessitating surgical treatment. These interventions were performed using laser ablation. Intervals between treatments shortened so that the shortest disease-free period was only 3 weeks. The last surgical excision was performed using surgical pliers under microscope control.

DNA from a biopsied specimen was isolated by means of a QIAamp DNA Mini Kit (tissue protocol) according to the manufacturer's handbook (QIAGEN, Hilden, Germany). Extracted DNA was amplified in a PTC 200 PCR thermocycler (MJ Research, Waltham, Massachusetts, USA). As an internal control, a 110 bp long fragment of the human β-globin gene was amplified using PC03/PC04 primers. Positive β-globin amplification showed that the sample contained enough DNA and that no PCR inhibitors were present. HPV DNA was detected by PCR using GP5+/GP6+ primers specific for the L1 region. Genotyping was performed by reverse line blot hybridisation (RLB), which permits the detection of 37 HPV types in a single assay.[1] Genotyping in our case showed HPV type 11.

Immunological tests found no defects in humoral or cellular immunity. ELISA was used to test for anti-HPV antibodies using virus-like particles composed of capsid antigen L1 produced in a baculovirus expressing system in insect cells. No antibodies were found against HPV types 6, 11, 16, 18, 31 or 33. All HPV diagnostic procedures were undertaken in the National Reference Laboratory for Human Papillomavirus in The Institute of Hematology and Blood Transfusion, Prague.

The progressive course of the patient's disease led us to consider stimulating an immune response to HPV-11 as this might have a better chance of achieving long term remission compared to standard adjuvant treatments (such as cidofovir). We chose vaccination using a commercially approved vaccine with anti HPV-11 activity (GARDASIL, Merck, New Jersey, USA; regional brand name SILGARD in the Czech Republic). We used a three-dose schedule of vaccination (at 0, 2 and 6 months), with the first dose given 1 month after the last surgical excision.

The overall clinical picture and the course of disease changed markedly after vaccination. Since administration of the first vaccine dose, the patient has not experienced any episodes of voice hoarsening and repeated laryngoscopic examinations have found no recurrent papillomas. Two months after the third (and last) vaccination we checked anti-HPV antibodies and detected anti HPV-6 and anti HPV-11 antibodies in plasma.

Subsequent immune status study showed increased levels of IgM antibodies (1.88 g/l; reference range 0.4–1.6 g/l) and normal absolute CD3+ (3.04×10^9/l; range 0.7–4.2), CD4+ (1.9×10^9/l; range 0.3–2.0) and CD8+ (0.93×10^9/l; range 0.3–1.8) T cell counts. At the time of writing, our patient has had no evidence of papillomatosis for 17 months. This is her longest disease-free period and her voice has regained normal physiological tone.

DISCUSSION

Recurrent laryngeal papillomatosis (RLP) in children is a rare, chronic and potentially devastating disease significantly affecting quality of life. Disappointing treatment results with laser surgery are not uncommon and encourage exploration of new therapeutic options. For decades non-specific immunotherapy with interferon α has been one of the few options. However, treatment with interferon α has a risk of lesion relapse after discontinuation of treatment. Furthermore, interferon treatment also has significant systemic toxicities.[2] Some evidence of the efficacy of antiviral agents as adjuvant agents in the management of RLP in

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children and/or adults has been also published.[3 4] Adjuvant intralesional treatment with bevacizumab showed some efficacy in prolonging the time between treatments and therefore reducing the number of treatments per year in children with severe RLP.[5] All described options have limited efficacy or significant toxicities and/or need surgical intervention.

Recent research has described the central importance of the CD4 T cell population in the control of HPV infection.[6] We were aware that treatment with HPV vaccine may not lead to a cellular immune response as most reports describe the vaccine as producing serum neutralising antibody to HPV major capside protein L1. Given the absence of anti HPV-11 antibodies in our patient, we decided to try to improve her HPV-11 antibody production and also to stimulate T cell-dependent specific anti-HPV immunity. Our evaluation of immune response after vaccination was limited to only general humoral and cell immunity together with evaluation of specific anti-HPV antibodies. Of course, this does not provide any proof of anti-HPV T cell activity. Moreover, a different surgical technique was used during the last intervention, so we do not know for definite which action was curative for this child.

Previously published case reports described stabilisation of RLP with small residual papilloma after HPV vaccination in a 2-year-old boy and in an adult.[7 8] As in these published cases, we also observed a fundamental change in disease course after vaccination with HPV vaccine, but in contrast to them, our patient was in RLP remission after the last surgical excision. To our knowledge, this disease remission lasting 17 months after HPV vaccination is the longest reported in the literature to date.

We demonstrated that vaccination using an off-label indication for an already approved drug can induce prolonged clinical remission of this otherwise devastating condition. Obviously, more extensive multicentre studies are needed to fully assess the potential benefit of this therapeutic approach.[7 9] While we await these studies, a centralised international database of similar cases would be a good first step towards understanding and monitoring this condition.

Competing interests None.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

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Safety, Efficacy, and Exposure–Response of Voriconazole in Pediatric Patients With Invasive Aspergillosis, Invasive Candidiasis or Esophageal Candidiasis

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Background: Data on safety and efficacy of voriconazole for invasive aspergillosis (IA) and invasive candidiasis/esophageal candidiasis (IC/EC) in pediatric patients are limited.

Methods: Patients aged 2–<18 years with IA and IC/EC were enrolled in 2 prospective open-label, non-comparative studies of voriconazole. Patients followed dosing regimens based on age, weight and indication, with adjustments permitted. Treatment duration was 6–12 weeks for IA patients, ≥14 days after last positive *Candida* culture for IC patients and ≥7 days after signs/symptoms resolution for EC patients. Primary analysis for both the studies was safety and tolerability of voriconazole. Secondary end points included global response success at week 6 and end of treatment (EOT), all-causality mortality and time to death. Voriconazole exposure–response relationship was explored.

Results: Of 53 voriconazole-treated pediatric patients (31 IA; 22 IC/EC), 14 had proven/probable IA, 7 had confirmed IC and 10 had confirmed EC. Treatment-related hepatic and visual adverse events, respectively, were reported in 22.6% and 16.1% of IA patients, and 22.7% and 27.3% of IC/EC patients. All-causality mortality in IA patients was 14.3% at week 6; no deaths were attributed to voriconazole. No deaths were reported for IC/EC patients. Global response success rate was 64.3% (week 6 and EOT) in IA patients and 76.5% (EOT) in IC/EC patients. There was no association between voriconazole exposure and efficacy; however, a slight positive association between voriconazole exposure and hepatic adverse events was established.

Conclusions: Safety and efficacy outcomes in pediatric patients with IA and IC/EC were consistent with previous findings in adult patients.

Key Words: voriconazole, aspergillosis, candidiasis, pediatric, exposure–response

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Aspergillus and *Candida* species are the predominant causes of invasive fungal infection in pediatric patients.[1] The incidence of invasive fungal infection has increased substantially in recent years, largely because of the increasing number of children at risk of acquiring these infections.[1]

Invasive aspergillosis (IA) is observed in children with compromised phagocytic function,[2–4] as well as in patients with hematologic malignancies and specific immunosuppression, and recipients of allogeneic stem cell and solid organ transplants.[4] Although the lungs are the most common infection site,[4] the central nervous system, cardiovascular system and other tissues may be infected because of hematogenous dissemination in severely immunocompromised patients.[5] Invasive candidiasis (IC) may present as catheter-associated candidemia, single-organ candidiasis or disseminated candidiasis, with or without candidemia.[6] Risk factors for IC include intensive care unit admission, neutropenia, malignant diseases[7] and congenital immunologic deficiencies.[8]

Voriconazole is a broad-spectrum triazole with activity against a wide range of yeasts and filamentous fungi. It is a substrate and inhibitor of the cytochrome P450 (CYP) isoenzymes CYP2C19, CYP2C9 and CYP3A4. Voriconazole exhibits nonlinear pharmacokinetics because of saturation of its metabolism; inter-individual variability in exposure is high.[9–11] In healthy adults, it has been demonstrated that CYP2C19 genotyping status, gender and age are key factors, which contribute to this variability.[12] In healthy adults, poor metabolizers of CYP2C19 have, on average, approximately 2–4-fold higher voriconazole levels than their homozygous extensive metabolizers and heterozygous extensive metabolizers counterparts, respectively, independent of ethnicity.[12,13] However, exposure of voriconazole varies widely within each genotype and overlaps considerably across genotypes.[12] Therefore, no dose adjustment based on CYP2C19 genotyping status is warranted in the current product label for voriconazole.

Although efficacy has been demonstrated in adults with IA, IC and esophageal candidiasis (EC),[14–16] published data on voriconazole use in children are limited.[10,11,17–20] Given the potentially life-threatening nature of invasive fungal infection, data on efficacy, safety and dosing of voriconazole in children will be of value to the medical community. Here, we evaluated safety, efficacy and exposure–response of voriconazole for the treatment of IA, IC and EC in pediatric patients using the recently revised dosing regimens in 2 prospective, open-label, noncomparative studies.

MATERIALS AND METHODS

Study A1501080 evaluated pediatric patients with IA (vori-IA study; NCT00836875), whereas Study A1501085 evaluated pediatric patients with IC/EC (vori-IC/EC study; NCT01092832). Both studies were conducted in compliance with the Declaration of Helsinki and International Conference on Harmonisation Good Clinical Practice Guidelines and were approved by the appropriate individual Institutional Review Boards for each study site. Investigators obtained written, informed consent from legally acceptable representatives and patient assent, where applicable.

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Study Design and Treatment

The vori-IA and vori-IC/EC studies were prospective, open-label, noncomparative phase 3 studies. The vori-IA study was conducted at 16 centers (Asia, Europe and North America) from 2009 to 2013; the vori-IC/EC study was conducted at 11 centers (Asia, Europe, North America) from 2010 to 2013.

In both studies, patients followed recently revised dosing regimens based on age, weight and indication (Table 1). These initial dosing regimens were based on an integrated population pharmacokinetic analysis of voriconazole data from children, adolescents and adults.[21] Patients initiated treatment with intravenous (IV) voriconazole and continued IV therapy until clinical improvement was observed. Treatment for IA and IC started with loading doses of 9 mg/kg every 12 hours (q12 h) for the first 24 hours for children (aged 2–<12 years) and young adolescents (aged 12–14 years, weighing <50 kg), followed by maintenance doses of 8 mg/kg q12 h. For all other adolescents (aged 12–<18 years, excluding 12–14-year olds weighing <50 kg), the loading doses were 6 mg/kg q12 h for the first 24 hours followed by maintenance doses of 4 mg/kg q12 h. Children with EC did not receive loading doses of IV voriconazole. Dosage for children (aged 2–<12 years) and young adolescents (aged 12–14 years, weighing <50 kg) began with 4 mg/kg q12 h. Dosage for all other adolescents (aged 12–<18 years, excluding 12–14 year-olds weighing <50 kg) began with 3 mg/kg q12 h.

Patients could switch to oral voriconazole after 1 week (vori-IA) or 5 days (vori-IC/EC) of IV therapy. In the vori-IA study, patients received voriconazole for ≥6 weeks, up to a maximum of 12 weeks. A minimum treatment duration of 6 weeks was chosen based on recent clinical observations that this duration is sufficient to evaluate clinical efficacy in patients receiving therapy for IA.[22] Duration of treatment was based on clinical improvement and improvement in radiologic findings. In the vori-IC/EC study, patients received voriconazole for ≥14 days after the last positive *Candida* culture from a normally sterile site (for IC) or ≥7 days after the resolution of clinical signs/symptoms (for EC), up to a maximum of 42 days. Patients had to return for the 1-month follow-up visit after end of treatment (EOT).

Dose Adjustments

Dose adjustments were made based on clinical response, tolerability or voriconazole plasma trough concentrations (C_{min}; collected before dosing on third day or later of IV therapy or after switching to oral therapy, as well as after each dose adjustment). Although no definitive relationship between voriconazole exposure and response has been established, provisional cut-off values of C_{min} were used to inform dose adjustment. It is of note that children have less accumulation in response to a given dose of voriconazole than adults because of their faster metabolism of voriconazole; as detailed in an earlier analysis, to achieve the same total exposure [ie, area under the curve from 0 to 12 h (AUC_{0-12})], the corresponding C_{min} in children is expected to be lower than that in adults.[20] Therefore, the minimum of target voriconazole C_{min} in children in this study was lower than that used in adults.

For all treatments, the dose could be reduced by 1 mg/kg steps (or 50 mg steps if 350 mg oral dose was used) if it exceeded 6 μg/mL. If C_{min} was too high (eg, >10 μg/mL), the investigator could reduce the dose by >1 mg/kg or 50 mg, as needed and temporary discontinuation of dosing (eg, 24-hour washout) was allowed to avoid further accumulation of voriconazole in the body.

For IA and IC treatment, the dose could be increased in 1 mg/kg steps if C_{min} was <0.5 μg/mL during IV therapy or increased in 1 mg/kg or 50 mg steps if C_{min} was <0.2 μg/mL during step-down oral therapy. For EC treatment, the dose could be increased in 1 mg/kg or 50 mg steps if C_{min} was <0.2 μg/mL during IV or oral therapy. Close monitoring of adverse events (AEs) was implemented when the dose was increased.

To make voriconazole concentration data available to the investigators within 72 hours of receiving samples, trough plasma samples (approximately 1 mL) were analyzed at designated reference laboratories or locally.

CYP2C19 Genotyping

Buccal swab samples were collected for CYP2C19 genotyping and analyzed at Pfizer Pharmacogenomics Laboratory (Groton, CT) using a published method.[20]

Patients

Inclusion Criteria

In the vori-IA study, eligible patients were aged 2–<18 years, immunocompromised with a clinically compatible illness and had proven, probable or possible IA based on European Organization for Research and Treatment of Cancer/Mycoses Study Group consensus definitions.[23] Patients enrolled with possible IA were assessed again to determine whether they had proven or probable IA based on tests done within 7 days of the first dose of study drug. Patients with rare molds (eg, *Scedosporium* or *Fusarium* species) were also eligible. In the vori-IC/EC study, eligible patients were aged 2–<18 years with confirmed IC/EC. Invasive candidiasis diagnosis was based on growth of *Candida* species or mycologic evidence indicative

TABLE 1. Initial Voriconazole Dosing Scheme by Age, Weight and Indication

	Loading Dose	Maintenance Dose	
	IV	IV	Switched to Oral Voriconazole
Children (aged 2–<12 yr) and young adolescents (aged 12–14 yr weighing <50 kg)			
IA/IC	9 mg/kg q12 h for first 24 h	8 mg/kg q12 h	9 mg/kg q12 h (maximum dose 350 mg)
EC	–	4 mg/kg q12 h	9 mg/kg q12 h (maximum dose 350 mg)
Adolescents (aged 12–<18 yr) excluding those aged 12–14 yr weighing <50 kg			
IA/IC	6 mg/kg q12 h for first 24 h	4 mg/kg q12 h	200 mg q12 h*
EC	–	3 mg/kg q12 h	200 mg q12 h

*At the investigator's discretion, an oral dose of 300 mg q12 h may be used in adolescents with IA.
EC indicates esophageal candidiasis; IA, invasive aspergillosis; IC, invasive candidiasis; IV, intravenous; q12h, every 12 hours.

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of *Candida* species and later confirmed as *Candida* species from a specimen obtained from a sterile site within 7 days (primary therapy) or 14 days (salvage therapy) of first voriconazole dose. Patients with clinical and/or radiologic findings consistent with disseminated disease and a positive *Candida* culture from a normally sterile site within previous 2 weeks of diagnosis were also eligible. Esophageal candidiasis diagnosis was based on the presence of clinical symptoms/lesions consistent with EC, or positive microscopy and/or mycologic culture for *Candida* species from brush/tissue biopsy of esophageal lesions within 7 days of enrollment.

Exclusion Criteria

The vori-IA study excluded patients with sarcoidosis, aspergilloma, allergic bronchopulmonary aspergillosis or chronic IA with the duration of symptoms or radiologic findings for >4 weeks before entry. Patients who received previous treatment or prophylaxis with systemic agents against *Aspergillus* species or systemic antifungal treatment for the current episode of IA or rare molds for >96 hours were also excluded. Patients were excluded from the vori-IC/EC study (for primary therapy) if they required treatment with another systemic antifungal agent or had >48 hours of antifungal therapy before first voriconazole dose.

Safety

In both studies, the primary end point was safety and tolerability of voriconazole, as determined by the rate of AEs and treatment discontinuations because of AEs. Adverse events were monitored by the study investigators from screening until the 1-month follow-up visit after EOT and were recorded and coded using the Medical Dictionary for Regulatory Activities (MedDRA, v16.0). Investigators assessed the causality of all AEs. An investigator's causality assessment was the determination of whether there was a reasonable possibility that the study drug caused or contributed to the AE. A serious adverse event (SAE) was defined as any untoward medical occurrence at any dose that resulted in death, was life-threatening (immediate risk of death), required inpatient hospitalization or prolonged hospitalization, resulted in significant or permanent disability/incapacity (substantial disruption of the ability to perform normal life functions) or resulted in congenital abnormality/birth defect.

Visual assessments were performed at baseline, and at weeks 1, 2, 4, 6 and 12 or EOT. In children aged ≥3 years, visual symptoms were assessed primarily by a visual questionnaire, by the Hardy–Rand–Rittler color vision test and by acuity and fixation indices of the Early Treatment Diabetic Retinopathy Study chart, used at the investigator's discretion. Patients with treatment-emergent visual AEs underwent ophthalmic fundoscopy, and all findings were recorded. Children aged <3 years had visual fixation assessed by the investigators. Liver function tests were monitored weekly up to week 6, at week 12 and at the 1-month follow-up visit. All clinically significant hepatic and other laboratory abnormalities were reported as AEs or SAEs, as appropriate. Potential Hy's Law cases were reported as SAEs and were defined as patients who had aspartate aminotransferase (AST) or alanine aminotransferase (ALT) and total bilirubin baseline values within the normal range who, following treatment, presented with AST or ALT 3 × the upper limit of normal (ULN) concurrent with a total bilirubin 2 × ULN with no evidence of hemolysis and alkaline phosphatase 2 × ULN. Alternatively, if patients with pre-existing ALT, AST, or total bilirubin values above the ULN, then presented with AST or ALT 2 × baseline values and 3 × ULN or 8 × ULN (whichever was smaller) or total bilirubin increased by 1 × ULN or ≥3 × ULN (whichever was smaller), this was also considered a serious hepatic AE.

Efficacy

Efficacy assessments were secondary end points and included global response (success rate) at week 6 (vori-IA) and EOT (vori-IA and vori-IC/EC), all-causality mortality, and time to death. In the vori-IA study, successful global response was defined as clinical resolution or improvement of signs/symptoms plus complete/partial resolution of radiologic findings. In the vori-IC/EC study, successful global response was defined as clinical cure/improvement plus confirmed/presumed microbiologic eradication.

Exposure–Response Analyses

Voriconazole concentration data from 48 patients (96 observations) were analyzed using a nonlinear mixed-effects model using NONMEM system (version 7.2). Individual exposure parameters [area under the curve from 0 to 12 hours ($AUC_{0–12}$) and C_{min}] were estimated based on the final pharmacokinetic model. Relationships between voriconazole exposures and key safety (hepatic, visual, psychiatric, skin and subcutaneous tissue AEs) and efficacy (global response at EOT) end points were assessed by graphical examination or using a logistic regression model (NONMEM). The model selection was based on goodness-of-fit criteria, which included basic diagnostic plots, precision of parameter estimates and the objective function value. The graphic processing of the data and NONMEM output was performed with R (version 2.12.2).

As there could be multiple AE observations per patient, both single-panel (without counting the frequency of AE occurrence in each patient) and multiple-panel (includes all AE observations) analysis approaches were utilized for hepatic and visual AE analysis, including both all-causality and treatment-related events. As there were only a few psychiatric disorders and skin and soft tissue disorders reported, a simple descriptive check was performed for these 2 types of AEs.

The following hepatic AE terms were included in the analysis: ALT increased or abnormal, AST increased or abnormal, γ-glutamyl transferase increased or abnormal, bilirubin increased, hyperbilirubinemia, transaminases increased, liver function tests abnormal, gallbladder disorder, hepatosplenomegaly, jaundice cholestatic, liver disorder and drug-induced liver injury. The following visual AE terms were included in the analysis: abnormal sensation in the eye, asthenopia, chromatopsia, diplopia, photophobia, visual impairment, vision blurred and visual acuity reduced. There were 5 all-causality psychiatric disorders: insomnia (n = 2), depression, affect lability, and intentional self-injury. There were 6 treatment-related skin and subcutaneous tissue disorders: dermatitis exfoliative, rash maculopapular, skin burning sensation, skin lesion and rash (n = 2).

Statistical Analyses

The safety population comprised all patients who received ≥1 voriconazole dose. The modified intent-to-treat population for efficacy evaluation comprised all patients with proven/probable IA, microbiologically confirmed IC, presumed EC (patients with neutropenia, thrombocytopenia or advanced HIV/AIDS concurrent with oral candidiasis) or microbiologically confirmed EC who received ≥1 voriconazole dose. Safety and efficacy data for both the studies were descriptive in nature; thus, no statistical testing was performed.

RESULTS

Patient Disposition and Baseline Demographics

Vori-IA Study

Thirty-one patients received voriconazole, of whom 16 completed the treatment and 25 completed the study (ie, returned for 1-month follow-up visit; patients who did not return for the 1-month follow-up visit were considered to have discontinued the study; Fig. 1). Patient demographics are presented in Table 2. Most patients (82.8%) had a recent history of neutropenia, and 17.2% were recipients of hematopoietic stem cell transplants (allogeneic: 13.8%, autologous: 3.4%). Median (range) duration of IV treatment

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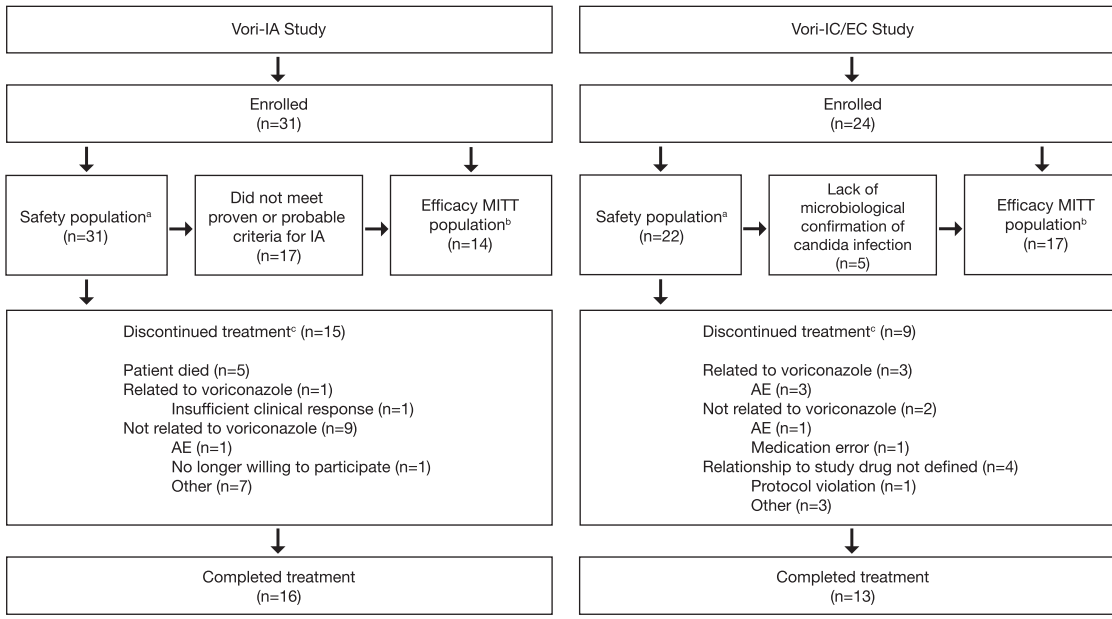

FIGURE 1. Patient disposition. [a]All patients who received ≥1 voriconazole dose. [b]All patients with proven/probable IA, microbiologically confirmed IC, presumed EC or microbiologically confirmed EC who received ≥1 voriconazole dose. [c]Patients who discontinued study treatment for any reason were not considered to have completed treatment. AE indicates adverse event; EC, esophageal candidiasis; IA, invasive aspergillosis; IC, invasive candidiasis; MITT, modified intent-to-treat.

TABLE 2. Patient Demographic Characteristics in the Vori-IA and Vori-IC/EC Studies (Safety Population)

	Vori-IA Study			Vori-IC/EC Study		
	2–<12 Yr (n = 11)	12–<18 Yr (n = 20)	Overall (n = 31)	2–<12 Yr (n = 14)	12–<18 Yr (n = 8)	Overall (n = 22)
Age in yr, mean (SD)	7.9 (2.3)	14.1 (1.7)	11.9 (3.5)	6.8 (2.9)	14.4 (1.7)	9.5 (4.5)
Sex, n (%)						
Female	4 (36.4)	11 (55.0)	15 (48.4)	8 (57.1)	6 (75.0)	14 (63.6)
Male	7 (63.6)	9 (45.0)	16 (51.6)	6 (42.9)	2 (25.0)	8 (36.4)
Race, n (%)						
White	3 (27.3)	8 (40.0)	11 (35.5)	5 (35.7)	5 (62.5)	10 (45.5)
Black	-	1 (5.0)	1 (3.2)	-	-	-
Asian	8 (72.7)	10 (50.0)	18 (58.1)	5 (35.7)	1 (12.5)	6 (27.3)
Other	-	1 (5.0)	1 (3.2)	4 (28.6)	2 (25.0)	6 (27.3)
Weight in kg, mean (SD)	26.7 (9.7)	50.1 (15.3)	41.7 (17.6)	23.9 (11.1)	54.6 (19.7)	35.1 (20.8)
Host factors for IA, n (%)						
Recent history of neutropenia	9 (81.8)	15 (83.3)*	24 (82.8)†	-	-	-
Hematopoietic stem cell transplant	2 (18.2)	3 (16.7)*	5 (17.2)†	-	-	-
Allogeneic	2 (18.2)	2 (11.1)*	4 (13.8)†	-	-	-
Autologous	-	1 (5.6)*	1 (3.4)†	-	-	-
Risk factors for IC/EC, n (%)						
Broad-spectrum antibiotics	-	-	-	12 (85.7)	7 (87.5)	19 (86.4)
Chemotherapy	-	-	-	12 (85.7)	7 (87.5)	19 (86.4)
Neutropenia	-	-	-	10 (71.4)	8 (100.0)	18 (81.8)
Central venous catheter	-	-	-	11 (78.6)	6 (75.0)	17 (77.3)
Duration of IV treatment in days, median (range)	8.0 (3–33)	8.5 (5–22)	8.0 (3–33)	6.5 (2–24)	8.0 (5–17)	7.0 (2–24)
Duration of oral treatment in days, median (range)	55.0 (2–78)	59.5 (8–81)	59.5 (2–81)	15.0 (3–37)	5.0 (2–8)	9.0 (2–37)
Duration of total treatment in days, median (range)	37.0 (3–85)	43.5 (5–90)	41.0 (3–90)	16.5 (2–42)	14.0 (6–17)	14.0 (2–42)

*n = 18; host factor case report form pages were not completed for 2 patients.
†n = 29; host factor case report form pages were not completed for 2 patients.
EC indicates esophageal candidiasis; IA, invasive aspergillosis; IC, invasive candidiasis; IV, intravenous; SD, standard deviation.

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(n = 31), oral treatment (n = 22) and total treatment was 8.0 (3–33) days, 59.5 (2–81) days and 41.0 (3–90) days, respectively. Eleven patients (35.5%) required dose reduction, and 3 patients (9.7%) had a dose increase.

Of 31 enrolled patients, 14 were diagnosed with proven/probable IA and 17 were diagnosed with possible IA. Baseline characteristics for those patients with proven/probable IA are presented in Table 3. All patients had a blood and lymphatic system disorder. Metabolism and nutrition disorders, neoplasms, gastrointestinal disorders and infections were common. The lungs were a site of infection in all patients. All identified pathogens were *Aspergillus* species.

Vori-IC/EC Study

Twenty-two patients received voriconazole, of whom 13 completed the treatment and 21 completed the study. Patient demographics are presented in Table 2. Most patients had a recent history of broad-spectrum antibiotic therapy (86.4%), chemotherapy (86.4%), neutropenia (81.8%) and central venous catheter use (77.3%). Median (range) duration of IV treatment (n = 22), oral treatment (n = 13) and total treatment was 7.0 (2–24) days, 9.0 (2–37) days and 14.0 (2–42) days, respectively. Three patients (13.6%) required dose reduction, and 3 patients (13.6%) had a dose increase.

TABLE 3. Patient Baseline Characteristics in the Vori-IA Study (MITT Population)

	Vori-IA Study		
	2–<12 Yr (n = 5)	12–<18 Yr (n = 9)	Overall (n = 14)
Most common (occurring in ≥5 patients) medical conditions by SOC, n (%)			
Blood and lymphatic system disorders	5 (100.0)	9 (100.0)	14 (100.0)
Metabolism and nutrition disorders	3 (60.0)	7 (77.8)	10 (71.4)
Neoplasms (benign, malignant, and unspecified)	3 (60.0)	7 (77.8)	10 (71.4)
Gastrointestinal disorders	3 (60.0)	6 (66.7)	9 (64.3)
Infections	4 (80.0)	5 (55.6)	9 (64.3)
General disorders and administration site conditions	1 (20.0)	6 (66.7)	7 (50.0)
Renal and urinary disorders	1 (20.0)	4 (44.4)	5 (35.7)
EORTC criteria for IA, n (%)			
Proven	2 (40.0)	6 (66.7)	8 (57.1)
Probable	3 (60.0)	3 (33.3)	6 (42.9)
Host factors, n (%)			
Recent history of neutropenia	3 (60.0)	7 (87.5)*	10 (76.9)†
Hematopoietic stem cell transplant	1 (20.0)	2 (25.0)*	3 (23.1)†
Autologous	0 (0.0)	1 (12.5)*	1 (7.7)†
Allogeneic	1 (20.0)	1 (12.5)*	2 (15.4)†
Myeloablative	1 (20.0)	2 (25.0)*	3 (23.1)†
Corticosteroid therapy	1 (20.0)	1 (12.5)*	2 (15.4)†
Other T-cell immunosuppressants	0	2 (25.0)*	2 (15.4)†
Site of infection, n (%)‡			
Lung	5 (100.0)	9 (100.0)	14 (100.0)
Sinus	-	2 (22.2)	2 (14.3)
Other	2 (40.0)	-	2 (14.3)
Baseline pathogen, n (%)§			
Aspergillus species (unidentified)	3 (60.0)	7 (77.8)	10 (71.4)
Aspergillus flavus	-	1 (11.1)	1 (7.1)
Aspergillus fumigates	-	2 (22.2)	2 (14.3)

*n = 8. In 1 patient, the host factor case report form was not completed as the patient's medical condition (suspected congenital cystic adenomatoid malformation) was not prespecified; the patient was included in the efficacy (MITT) population based on recent lung lobectomy, lung tissue biopsy positive for *Aspergillus* species, positive serum galactomannan and pleural effusion.

†n = 13.

‡Patients could have multiple sites at baseline.

§Four patients did not have *Aspergillus* species isolated but were included in the efficacy (MITT) population based on the following: 3 patients had a positive serum galactomannan, 1 patient had sputum gram-stain sample positive for hyphae.

EORTC indicates European Organisation for Research and Treatment of Cancer; IA, invasive aspergillosis; MITT, modified intent-to-treat; SOC, system organ class.

TABLE 4. Patient Baseline Characteristics in the Vori-IC/EC Study (MITT Population)

	Vori-IC/EC Study		
	2–<12 Yr (n = 9)	12–<18 Yr (n = 8)	Overall (n = 17)
Most common (occurring in ≥5 patients) medical conditions by SOC, n (%)			
Neoplasms (benign, malignant and unspecified)	8 (88.9)	7 (87.5)	15 (88.2)
Blood and lymphatic system disorders	6 (66.7)	8 (100.0)	14 (82.4)
Infections	6 (66.7)	6 (75.0)	12 (70.6)
Metabolism and nutrition disorders	5 (55.6)	5 (62.5)	10 (58.8)
Gastrointestinal disorders	3 (33.3)	4 (20.0)	7 (41.2)
General disorders and administration site conditions	3 (33.3)	3 (37.5)	6 (35.3)
Nervous system disorders	1 (11.1)	5 (62.5)	6 (35.3)
Psychiatric disorders	-	5 (62.5)	5 (29.4)
Respiratory, thoracic, and mediastinal disorders	1 (11.1)	4 (20.0)	5 (29.4)
Fungal diagnosis, n (%)			
IC	7 (77.8)	-	7 (41.2)
Primary therapy	5 (55.6)	-	5 (29.4)
Salvage therapy	2 (22.2)	-	2 (11.8)
EC	2 (22.2)	8 (100.0)	10 (58.8)
Primary therapy	2 (22.2)	6 (75.0)	8 (47.1)
Salvage therapy	-	2 (25.0)	2 (11.8)
Candida risk factors, n (%)			
Chemotherapy	7 (77.8)	7 (87.5)	14 (82.4)
Use of broad-spectrum antibiotics	7 (77.8)	7 (87.5)	14 (82.4)
Neutropenia	5 (55.6)	8 (100.0)	13 (76.5)
Use of central venous catheter	7 (77.8)	6 (75.0)	13 (76.5)
Clinical sepsis	5 (55.6)	5 (62.5)	10 (58.8)
Immunosuppressive therapy	5 (55.6)	5 (62.5)	10 (58.8)
Mucosal colonization	5 (55.6)	5 (62.5)	10 (58.8)
Use of systemic corticosteroids/other immunosuppressive drugs	6 (66.7)	3 (37.5)	9 (52.9)
Multifocal colonization	2 (22.2)	3 (37.5)	5 (29.4)
Total parenteral nutrition	3 (33.3)	2 (25.0)	5 (2.4)
Length of ICU stay >4 d, n (%)	2 (22.2)	2 (25.0)	4 (23.5)
Surgery	3 (33.3)	-	3 (17.6)
Abdominal surgery	2 (22.2)	-	2 (11.8)
Other	1 (11.1)	-	1 (5.9)
Site of infection, n (%)*			
Esophagus	2 (22.2)	8 (100.0)	10 (58.8)
Oropharynx	3 (33.3)	5 (62.5)	8 (47.1)
Blood	7 (77.8)	-	7 (41.2)
Catheter	2 (22.2)	-	2 (11.8)
Left eye	1 (11.1)	-	1 (5.9)
Lung	1 (11.1)	-	1 (5.9)
Right eye	1 (11.1)	-	1 (5.9)
Skin (unspecified)	1 (11.1)	-	1 (5.9)
Baseline pathogen, n (%)†			
Candida albicans	4 (44.4)	8 (100.0)	12 (70.6)
Candida tropicalis	3 (33.3)	-	3 (17.6)
Candida glabrata	1 (11.1)	-	1 (5.9)
Candida parapsilosis	1 (11.1)	-	1 (5.9)

*Patients could have multiple sites at baseline.

†Patients could have multiple organisms at baseline.

EC indicates esophageal candidiasis; IC, invasive candidiasis; ICU, intensive care unit; MITT, modified intent-to-treat; SOC, system organ class.

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Of 22 enrolled patients, 7 had confirmed IC and 10 had confirmed EC (the remaining 5 enrolled patients lacked microbiologic confirmation of *Candida*), with baseline characteristics presented in Table 4. The most common medical conditions were neoplasms, blood and lymphatic system disorders, infections and metabolism and nutrition disorders. The esophagus, oropharynx and blood were the most common sites of infection, and infection was related to central venous catheter use in 2 patients (data not shown). Most patients had infection caused by *Candida albicans*.

Safety

Vori-IA Study

A safety summary is presented in Table 5. Sixteen of 31 patients experienced 35 treatment-related AEs, most commonly blurred vision (n = 3) and photophobia, increased ALT, abnormal liver function test and transaminases increased (n = 2 each). Most treatment-related AEs were mild or moderate in severity. Treatment-related hepatic AEs were experienced by 7 patients (22.6%), and except for 1 patient with severe drug-induced liver injury (discussed below), all were mild or moderate in severity. Treatment-related visual AEs were reported by 5 patients (16.1%) and were mild in severity. Four patients (12.9%) reported treatment-related skin AEs [exfoliative dermatitis (n = 1), maculopapular rash (n = 1), skin burning sensation (n = 1) and skin lesion (n = 1)], which were all mild in severity, and only 1 patient reported any psychiatric treatment-related AE (insomnia; data not shown). Serious adverse events were experienced by 15 of 31 patients. Two SAEs were considered treatment related. Specifically, an 11-year-old girl experienced acute renal failure on day 34. The patient also received concomitant treatment with other medications, including ganciclovir (days 7–36) and vancomycin (days 28–29), while receiving treatment with the study drug. On day 32, the patient switched from IV to oral voriconazole and continued treatment for an additional 5 days. The patient died on day 38 due to sepsis. A case of drug-induced liver injury leading to discontinuation was reported on day 40 in a 14-year-old boy; this patient's underlying medical conditions at baseline included acute lymphocytic leukemia relapse, febrile neutropenia, herpes zoster oticus, hyperbilirubinemia, hypocalcemia, hypokalemia, hypomagnesemia, mucosal inflammation, pancytopenia, pneumonia, renal tubular disorder, rhinitis, sinusitis and thrombophlebitis. On day 40, the patient was hospitalized

TABLE 5. Summary of Safety Data From the Vori-IA Study

	Vori-IA Study					
	2–<12 Yr (n = 11)		12–<18 Yr (n = 20)		Overall* (n = 31)	
	All-Causality	Treatment-Related	All-Causality	Treatment-Related	All-Causality	Treatment-Related
AEs, n	86	7	195	28	281	35
Patients with AEs, n (%)	11 (100.0)	5 (45.5)	19 (95.0)	11 (55.0)	30 (96.8)	16 (51.6)
Hepatic AEs, n (%)	-	-	8 (40.0)	7 (63.6)	8 (25.8)	7 (22.6)
ALT increased	-	-	2 (10.0)	2 (10.0)	2 (6.5)	2 (6.5)
Liver function test abnormal	-	-	2 (10.0)	2 (10.0)	2 (6.5)	2 (6.5)
Transaminases increased	-	-	2 (10.0)	2 (10.0)	2 (6.5)	2 (6.5)
AST increased	-	-	1 (5.0)	1 (5.0)	1 (3.2)	1 (3.2)
Blood bilirubin increased	-	-	1 (5.0)	1 (5.0)	1 (3.2)	1 (3.2)
Drug-induced liver injury	-	-	1 (5.0)	1 (5.0)	1 (3.2)	1 (3.2)
Jaundice cholestatic	-	-	1 (5.0)	-	1 (3.2)	-
Visual AEs, n (%)	3 (27.3)	1 (9.1)	6 (30.0)	4 (20.0)	9 (29.0)	5 (16.1)
Vision blurred	-	-	3 (15.0)	3 (15.0)	3 (9.7)	3 (9.7)
Visual impairment	-	-	2 (5.0)	1 (5.0)	2 (6.5)	1 (3.2)
Photophobia	1 (9.1)	1 (9.1)	1 (5.0)	1 (5.0)	2 (6.5)	2 (6.5)
Conjunctivitis	-	-	2 (10.0)	-	2 (6.5)	-
Abnormal sensation in the eye	-	-	1 (5.0)	1 (5.0)	1 (3.2)	1 (3.2)
Asthenopia	-	-	1 (5.0)	1 (5.0)	1 (3.2)	1 (3.2)
Chromatopsia	-	-	1 (5.0)	1 (5.0)	1 (3.2)	1 (3.2)
Diplopia	-	-	1 (5.0)	1 (5.0)	1 (3.2)	1 (3.2)
Cataract	-	-	1 (5.0)	-	1 (3.2)	-
Conjunctival hemorrhages	1 (9.1)	-	-	-	1 (3.2)	-
Dry eye	1 (9.1)	-	-	-	1 (3.2)	-
Eye discharge	1 (9.1)	-	-	-	1 (3.2)	-
Eye irritation	-	-	1 (5.0)	-	1 (3.2)	-
Eye pain	-	-	1 (5.0)	-	1 (3.2)	-
SAEs, n (%)	6 (54.5)	1 (9.1)	9 (45.0)	1 (5.0)	15 (48.4)	2 (6.5)
Treatment discontinuation, n (%)	6 (54.5)	1 (9.1)	9 (45.0)	-	15 (48.4)	1 (3.2)
AEs	1 (9.1)	-	-	-	1 (3.2)	-
Insufficient clinical response	1 (9.1)	1 (9.1)	-	-	1 (3.2)	1 (3.2)
No longer willing to participate	-	-	1 (5.0)	-	1 (3.2)	-
Patient died	3 (27.3)	-	2 (10.0)	-	5 (16.1)	-
Other	1 (9.1)†	-	6 (30.0)‡	-	7 (22.6)	-
Study discontinuation, n (%)	3 (27.3)	-	3 (15.0)	-	6 (19.4)	-
Patient died	3 (27.3)	-	2 (10.0)	-	5 (16.1)	-
No longer willing to participate	-	-	1 (5.0)	-	1 (3.2)	-

*All patients received at least 1 dose of voriconazole. In the vori-IA study the median (range) duration of IV treatment (n = 31), oral treatment (n = 22) and total treatment was 8.0 (3–33) days, 59.5 (2–81) days and 41.0 (3–90) days, respectively.

†Visual testing was not completed at screening and day 7.

‡Addition of another antifungal medication for additional coverage based on computed tomography findings and continued positive galactomannan with increasing titers (n = 1); IA not approved (n = 1); no proven or probable IA (n = 1); IA not identified, relapsing of lymphoma (n = 1); no longer possible IA (proven *Candida tropicalis* infection; n = 1); patient diagnosed with bacterial lung infection.

AE indicates adverse event; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IA, invasive aspergillosis; SAE, severe adverse event.

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TABLE 6. Summary of Safety Data from the Vori-IC/EC Study (Safety Population)

	Vori-IC/EC Study					
	2–<12 Yr (n = 14)		12–<18 Yr (n = 8)		Overall* (n = 22)	
	All-Causality	Treatment-Related	All-Causality	Treatment-Related	All-Causality	Treatment-Related
AEs, n	78	13	35	5	113	18
Patients with AEs, n (%)	13 (92.9)	8 (57.1)	6 (75.0)	3 (37.5)	19 (86.4)	11 (50.0)
Hepatic AEs, n (%)	6 (42.9)	5 (35.7)	1 (12.5)	-	7 (31.8)	5 (22.7)
ALT abnormal	3 (21.4)	1 (7.1)	-	-	2 (9.1)	1 (4.5)
ALT increased	1 (7.1)	1 (7.1)	-	-	1 (4.5)	1 (4.5)
AST abnormal	1 (7.1)	1 (7.1)	-	-	1 (4.5)	1 (4.5)
AST increased	1 (7.1)	1 (7.1)	-	-	1 (4.5)	1 (4.5)
GGT abnormal	2 (14.3)	1 (7.1)	-	-	2 (9.1)	1 (4.5)
Hepatic enzyme increased	1 (7.1)	1 (7.1)	-	-	1 (4.5)	1 (4.5)
Hyperbilirubinemia	1 (7.1)	1 (7.1)	-	-	1 (4.5)	1 (4.5)
Liver disorder	1 (7.1)	1 (7.1)	-	-	1 (4.5)	1 (4.5)
Blood ALP abnormal	1 (7.1)	-	-	-	1 (4.5)	-
Gallbladder disorder	1 (7.1)	-	-	-	1 (4.5)	-
GGT increased	-	-	1 (12.5)	-	1 (4.5)	-
Hepatosplenomegaly	1 (7.1)	-	-	-	1 (4.5)	-
Jaundice	1 (7.1)	-	-	-	1 (4.5)	-
Visual AEs, n (%)	6 (42.9)	3 (21.4)	3 (37.5)	3 (37.5)	9 (40.9)	6 (27.3)
Photophobia	2 (14.3)	2 (14.3)	1 (12.5)	1 (12.5)	3 (13.6)	3 (13.6)
Conjunctivitis	1 (7.1)	1 (7.1)	-	-	1 (4.5)	1 (4.5)
Eye pruritus	-	-	1 (12.5)	1 (12.5)	1 (4.5)	1 (4.5)
Retinal disorder	-	-	1 (12.5)	1 (12.5)	1 (4.5)	1 (4.5)
Amaurosis	1 (7.1)	-	-	-	1 (4.5)	-
Corneal opacity	1 (7.1)	-	-	-	1 (4.5)	-
Eyelid disorder	1 (7.1)	-	-	-	1 (4.5)	-
Visual acuity reduced	1 (7.1)	-	-	-	1 (4.5)	-
SAEs, n (%)	2 (14.3)	-	1 (12.5)	1 (12.5)	3 (13.6)	1 (4.5)
Treatment discontinuation, n (%)	7 (50.0)	2 (14.3)	2 (25.0)	1 (12.5)	9 (40.9)	3 (13.6)
AEs	2 (14.3)	2 (14.3)	2 (25.0)	1 (12.5)	4 (18.2)	3 (13.6)
Medication error	1 (7.1)	-	-	-	1 (4.5)	-
Protocol violation	1 (7.1)	-	-	-	1 (4.5)	-
Other	3 (21.4)†	-	-	-	3 (13.6)	-
Study discontinuation, n (%)	1 (7.1)	-	-	-	1 (4.5)	-
Lack of confirmation of *Candida* infection	1 (7.1)	-	-	-	1 (4.5)	-

*All patients received at least one dose of voriconazole. In the vori-IC/EC study, the median (range) duration of IV treatment (n = 22), oral treatment (n = 13), and total treatment was 7.0 (2–24) days, 9.0 (2–37) days, and 14.0 (2–42) days, respectively.

†Lack of confirmation of *Candida* infection.

AE indicates adverse events; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; EC, esophageal candidiasis; GGT, γ-glutamyl transferase; IC, invasive candidiasis; IV, intravenous; SAE, severe adverse event.

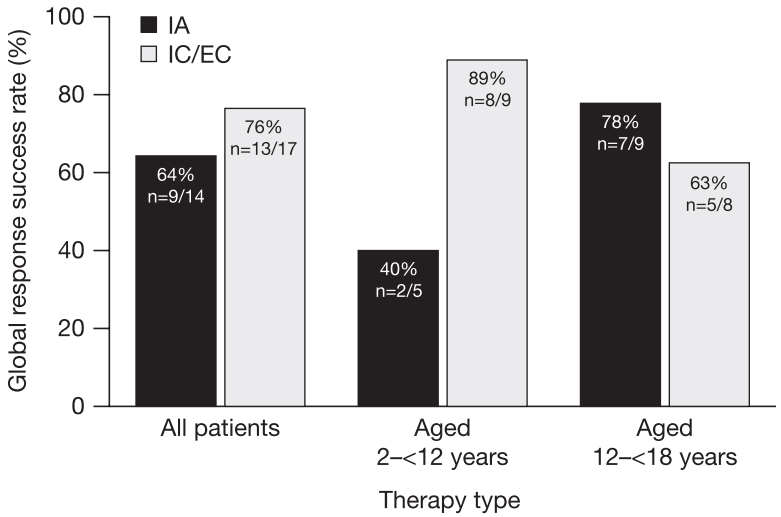

FIGURE 2. Global response success rates at EOT in patients with IA and IC/EC (MITT population). EC indicates esophageal candidiasis; EOT, end of treatment; IA, invasive aspergillosis; IC, invasive candidiasis; MITT, modified intent-to-treat.

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for severe muscle weakness and fever. At that time, the patient's blood bilirubin was 6.4 mg/dL, AST 694 IU/L and ALT 684 IU/L. The patient was also diagnosed with steroid-related muscle weakness and parainfluenza type 1 bronchitis. The patient completed voriconazole therapy for the treatment of IA on day 40. Liver function tests returned to normal on day 64 (24 days after last voriconazole dose).

Fifteen patients discontinued treatment. Only 1 patient (7-year-old male) discontinued treatment because of an AE; this patient discontinued on day 3 because of an SAE of sepsis (unrelated to voriconazole) and recovered by day 9. One treatment discontinuation was considered to be treatment related (insufficient clinical response); 6 patients were subsequently discontinued from the study for other reasons.

Vori-IC/EC Study

A safety summary is presented in Table 6. Eleven of 22 patients experienced 18 treatment-related AEs, most commonly photophobia (n = 3). Most treatment-related AEs were mild or moderate. Treatment-related hepatic AEs were reported in 5 patients (22.7%) and were mild or moderate in severity except for 1 case of severe liver disorder. Treatment-related visual AEs were reported by 6 patients (27.3%) and were mild or moderate in severity. Only 2 patients (9.1%) reported any treatment-related skin AEs [rash (=2); data not shown], which were both mild in severity; no psychiatric treatment-related AEs were observed. Serious adverse events were experienced by 3 of 22 patients; 1 SAE (EC patient), recorded as progression of suspected splenic candidiasis later confirmed by biopsy, was considered treatment related. Splenic candidiasis progressed to the kidneys and eye, despite systemic voriconazole treatment. Subsequent use of lipid amphotericin B and micafungin treatment did not lead to improvement; however, neutrophil reconstitution in addition to micafungin and posaconazole treatment led to remission on day 390 (373 days after last voriconazole dose).

Nine patients discontinued the treatment. Four patients discontinued the treatment because of AEs and, of these, 3 discontinued because of treatment-related AEs. Specifically, a 9-year-old female with IC (salvage) and medical history of pancreatic tumor, hyperbilirubinemia and heart failure permanently discontinued treatment on

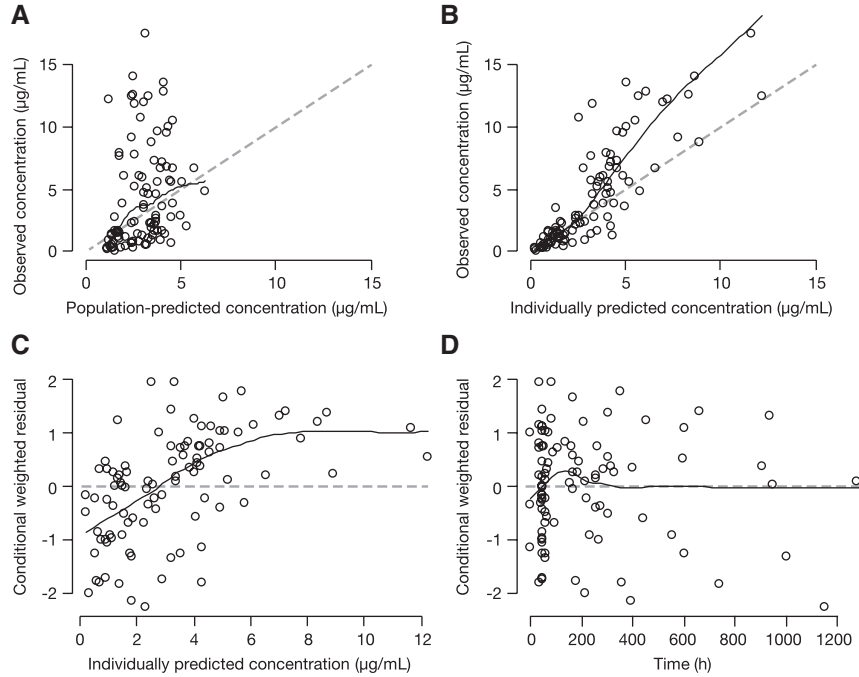

FIGURE 3. Basic goodness-of-fit plots for the final pharmacokinetic model, showing: observed concentrations versus population predicted concentrations (A); observed concentrations versus individually predicted concentrations (B); conditional weighted residuals versus individually predicted concentrations (C); conditional weighted residuals versus time (D). Open circles represent observed data; the dashed line represents the line of identity or unity; the solid line represents the local regression smooth line (loess smooth). The closer the smooth line is to the line of identity or unity, the more robust the model fit.

TABLE 7. Summary of Estimated Voriconazole Exposures in Pediatric Patients Based on Final Pharmacokinetic Model

	Voriconazole AUC_{0-12} (μg·h/mL)		Voriconazole C_{min} (μg/mL)	
Children (n = 21)				
Regimen	8 mg/kg IV q12 h	9 mg/kg oral q12 h*	8 mg/kg IV q12 h	9 mg/kg oral q12 h*
Geometric mean (CV%)	49.63 (57)	46.86 (60)	2.65 (77)	3.56 (64)
Median (range)	51.54 (20.67–171.08)	45.66 (19.84–170.76)	2.95 (0.69–12.67)	3.48 (1.39–13.86)
Young adolescents aged 12–14 yr weighing <50 kg (n = 10)				
Regimen	8 mg/kg IV q12 h	9 mg/kg oral q12 h*	8 mg/kg IV q12 h	9 mg/kg oral q12 h*
Geometric mean (CV%)	54.91 (40)	50.57 (43)	3.0 (52)	3.86 (46)
Median (range)	68.24 (20.35–85.79)	62.02 (19.54–82.44)	4.19 (0.66–5.61)	4.84 (1.36–6.51)
All other adolescents (n = 17)				
Regimen	4 mg/kg IV q12 h	200 mg oral q12 h	4 mg/kg IV q12 h	200 mg oral q12 h
Geometric mean (CV%)	37.28 (59)	27.72 (65)	2.18 (75)	2.15 (67)
Median (range)	33.78 (17.7–110.05)	25.07 (8.89–79.36)	1.97 (0.76–8.27)	1.94 (0.65–6.46)

*Maximum oral dose was not to exceed 350 mg q12 h.

AUC_{0-12} indicates area under the curve from 0 to12 hours; C_{min}, minimum plasma concentration (trough); CV%, coefficient of variation in percentage.

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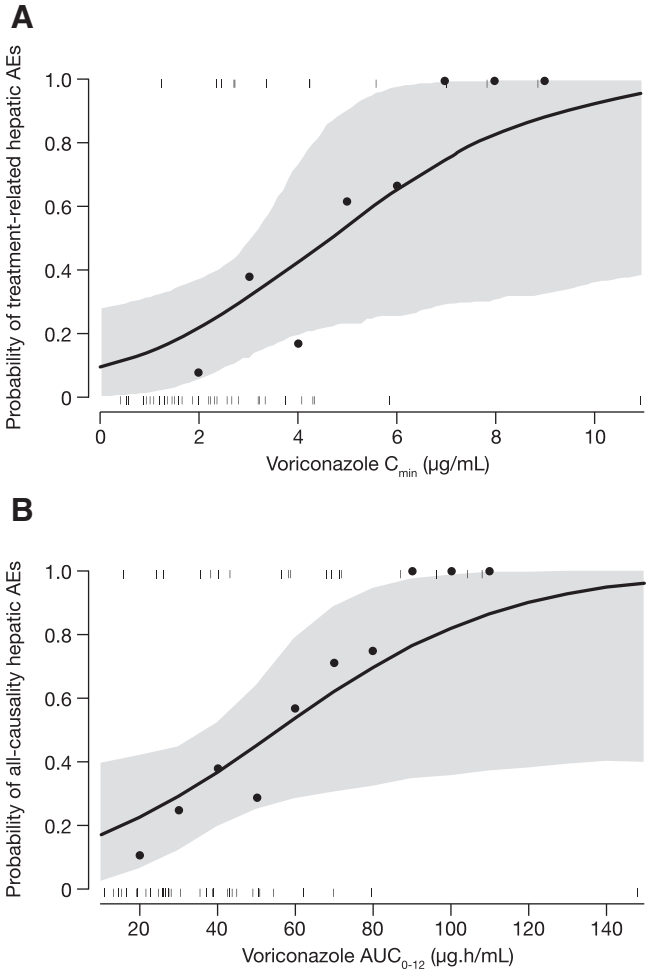

FIGURE 4. Observed and model-predicted probability of treatment-related hepatic AEs versus voriconazole C_{min} (A) and all-causality hepatic AEs versus voriconazole AUC_{0-12} (B) using multiple-panel data. "|" symbols represent observed individual data (AE present = 1, AE absent = 0); solid circles represent the observed probability of an AE at each concentration level (note: individual concentration values were rounded up to the next integral value for summary purposes). The line and the corresponding band represent the population-predicted probability and its 95% CI (computed with 1000 bootstrap). A wide 95% CI indicates low precision on the probability prediction. AE indicates adverse event; AUC_{0-12}, area under the curve from 0 to 12 hours; CI, confidence interval; C_{min}, minimum plasma concentration (trough).

day 12 after developing moderate hyperbilirubinemia, which resolved on day 17. A 5-year-old male with EC and medical history of acute lymphocytic leukemia, chemotherapy-related anemia, antithrombin III deficiency and hepatomegaly permanently discontinued the treatment on day 23 after developing severe liver disorder. On day 23, blood bilirubin was 2.1 mg/dL, AST 661.2 IU/L and ALT 282 IU/L. The event resolved by day 27 (4 days after last voriconazole dose). Concomitant medications taken within 2 weeks of the event of severe liver disorder included cytarabine, cyclophosphamide, pegaspargase, methotrexate and tioguanine. A 12-year-old girl with EC permanently discontinued the treatment on day 17 after developing severe progression of suspected splenic candidiasis as described above. One patient lost to follow-up was discontinued from the study.

Efficacy

Vori-IA Study

The week 6 global success rate in patients with proven/probable IA (n = 14) was 64.3% [95% confidence interval (CI): 35.1–87.2] and was sustained at EOT. Success rates were numerically greater for adolescents aged 12–<18 years [77.8% (95% CI: 40.0–97.2)] versus children aged 2–<12 years [40.0% (95% CI: 5.3–85.3)]. EOT global response failures included an observed failure in 1 patient, indeterminate result in 1 patient and missing data in 3 patients. Four deaths due to septic shock (n = 3) and ruptured mycotic aneurysm (n = 1) were reported before week 6 (up to day 63), and 1 death due to acute lymphocytic leukemia was reported on day 75. There were 2 deaths in modified intent-to-treat patients aged <12 years; none were attributed to voriconazole. One patient died on day 30 and the other died on day 38.

Vori-IC/EC Study

EOT global success rate in patients with IC/EC (n = 17) was 76.5% (95% CI: 50.1–93.2). EOT global success rates were 88.9% (95% CI: 51.7–99.7) for patients aged 2–<12 years and 62.5% (24.5, 91.5) for those aged 12–<18 years. Global response for IC patients (n = 7) included success in 6 patients and indeterminate result in 1 patient. Global response for EC patients (n = 10) included success in 7 patients, failure in 1 patient and indeterminate results in 2 patients. Two EC patients with a successful EOT global response had recurrence of EC (14 and 16 days after last voriconazole dose). One EC patient with a successful EOT global response developed suspected splenic candidiasis during therapy. EOT global response success rates by therapy and baseline pathogen are presented in Figure 2. No patients died by the 1-month follow-up visit.

Exposure–Response Analyses

For all age groups, a 2-compartment pharmacokinetic model with first-order absorption and linear elimination reasonably described voriconazole data, with the caveat of some underestimation of high concentrations, as shown in the basic diagnostic plots (Fig. 3). These plots showed that the data points were generally distributed symmetrically across the line of identity or line of unity, although many data points appeared to be widely spread, and several higher concentration data points were skewed from the line of identity or unity. This is not unexpected given the sparse data from phase 3 studies.

The equations for the final pharmacokinetic model are presented below, and interindividual variability was estimated for clearance only, given the limited concentration data.

$$\mathrm{CL} = \theta_{\mathrm{CL}} \left(\mathrm{WT} / 70\right)^{0.75}$$
$$V_2 = \theta_{V2}\, \mathrm{WT} / 70$$
$$V_3 = \theta_{V3}\, \mathrm{WT} / 70$$
$$Q = \theta_{Q} \left(\mathrm{WT} / 70\right)^{0.75}$$
$$\mathrm{logit}\left(F1\right) = \theta_{F1}$$
$$k_a = \theta_{ka}$$
$$\mathrm{Rate} = \theta_{\mathrm{rate}}$$

CL indicates linear clearance; V_2, central volume of distribution; V_3, peripheral volume of distribution; Q, intercompartmental clearance; $F1$, oral bioavailability; k_a, first-order absorption rate constant; rate, infusion rate used to estimate voriconazole concentration from prior treatment; and θ, estimate of fixed effects in NONMEM.

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At matching doses, voriconazole exposures in children and young adolescents with low body weight were comparable with those in heavier or older adolescents, given the large interindividual variability (Table 7). Although average voriconazole exposures tended to be greater in CYP2C19 poor metabolizers (n = 3) and heterozygous extensive metabolizers (n = 12) than homozygous extensive metabolizers (n = 17), substantial overlap in exposure distributions was seen across groups because of large interindividual variability (data on file; 16 patients did not have genotyping information available).

An association between increased voriconazole exposures ($AUC_{0–12}$ and C_{min}) and treatment-related hepatic AEs was established (Fig. 4A). For all-causality hepatic AEs, the association was only related to voriconazole $AUC_{0–12}$ but not C_{min} (Fig. 4B). The wide 95% CIs for the population predictions of probability of hepatic AE occurrence as a function of voriconazole exposure reflect the large uncertainty of the prediction (Fig. 4A and B). Note that this positive association was identified only when multiple-panel data (all AE occurrences) were analyzed. When single-panel data (without counting AE frequency in each patient) were analyzed, this positive association diminished for both treatment-related and all-causality hepatic AEs. No associations between voriconazole exposures and visual AEs, psychiatric or skin and subcutaneous tissue disorders were identified.

Given the limited sample size and high success rate, no association between voriconazole exposures and efficacy was established for IA and EC patients (Fig. 5A and B). All patients with IC for whom exposure data were available (n = 6) had global success at EOT; the exposure–response analysis was not performed because of the lack of failure cases. The average voriconazole $AUC_{0–12}$ in IC patients ranged from 27.2 to 62 µg·h/mL, and average C_{min} ranged from 1.09 to 4.32 µg/mL.

DISCUSSION

These data suggest that voriconazole is generally effective in pediatric patients with IA and IC/EC, with a favorable risk–benefit balance. Overall, the safety of voriconazole in this small number of pediatric patients was similar to the known safety profile in adults. Pediatric patients had a numerically greater frequency of hepatic-related AEs associated with liver enzyme elevations; however, the nature and severity of hepatic AEs in the pediatric population was similar to that seen in adults. Hepatic AEs (all-causality and treatment related) in the vori-IA study only occurred in patients aged 12–<18 years, whereas most hepatic AEs in the vori-IC/EC study occurred in patients aged 2–<12 years. Because visual disturbances are known side effects of voriconazole use in adults, visual symptoms were

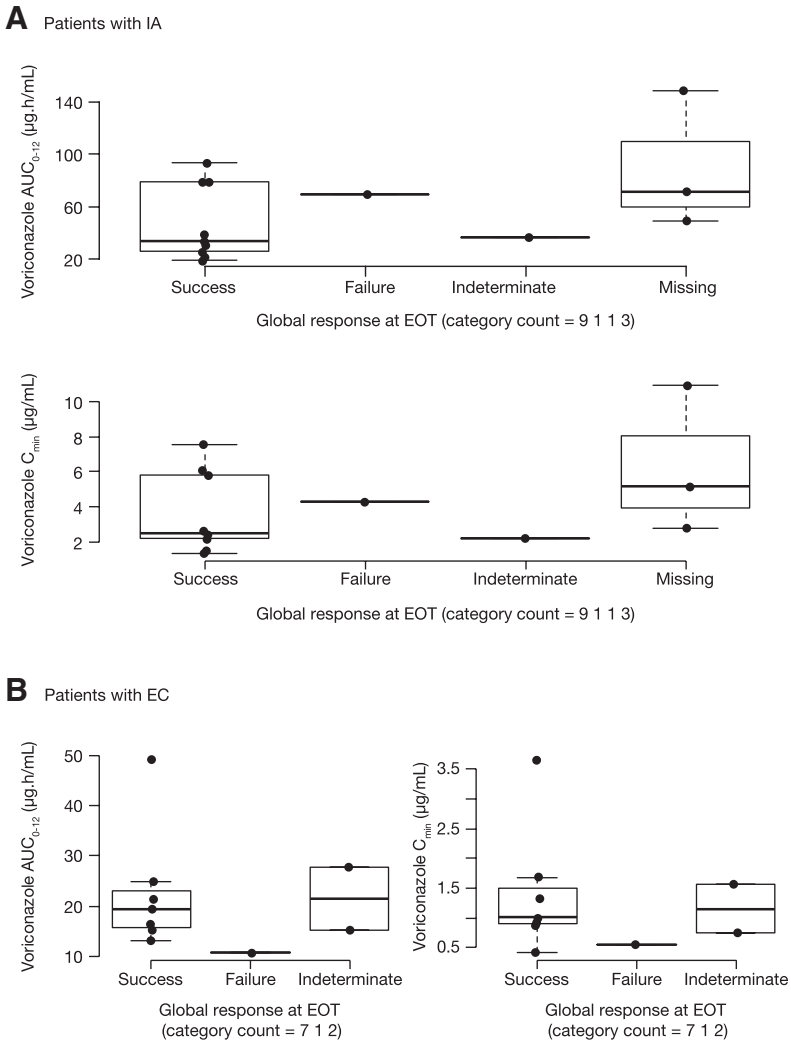

FIGURE 5. EOT global response versus voriconazole $AUC_{0–12}$ and C_{min} in patients with IA (A) and patients with EC (B). Horizontal center line represents the median; box represents the interquartile distance; whiskers represent ≤1.5 × interquartile range; solid circles represent the estimated individual exposure parameters. $AUC_{0–12}$ indicates area under the curve from 0 to 12 hours; C_{min}, minimum plasma concentration (trough); EC, esophageal candidiasis; EOT, end of treatment; IA, invasive aspergillosis.

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closely monitored throughout these studies. However, whether the tests used accurately assess visual AEs in children is unclear. It may be the case that children are unable to accurately report visual symptoms using these tests, instead, any visual disturbances manifest themselves as atypical behaviors. Of note, we did attempt to assess behavioral change in patients by administering the visual questionnaire, but no clear pattern of change was observed.

End of treatment global success rate in pediatric patients with IA was 64.3% (n = 9/14), similar to that seen in the adult therapeutic IA study (52.8%; n = 76/144) at 12 weeks.[15] In addition, EOT global success rates in pediatric patients with IC and EC were 85.7% (n = 6/7) and 70.0% (n = 7/10; indeterminate: n = 2/10), respectively, and comparable with those reported in the adult therapeutic studies for IC (65.3%; n = 162/248) and EC (98.3%; n = 113/115).[14,16] In the IA study, the success rate was numerically greater in patients aged 12–<18 years (77.8%) than in patients aged 2–<12 years (40.0%). In the IC/EC study, the reverse was true with greater success rate in patients aged 2–<12 years (88.9%) than in patients aged 12–<18 years (62.5%). However, any interpretation of these data is limited by the small subgroup sample sizes and by the open-label, non-comparative design of the presented studies.

Compared with the previously developed pharmacokinetic model for immunocompromised pediatric patients,[21] this model was simplified by removing the nonlinear component of clearance, without substantial degradation of model performance. The model fit voriconazole trough concentrations well although the absorption phase was poorly estimated, which was not unexpected as limited concentration data were available (particularly at absorption phase). On the basis of the totality of the model performance metrics, the simplified model was deemed acceptable to provide individual voriconazole exposure estimates.

Typical voriconazole clearance in these pediatric patients was greater than that in adults with IA (7.79 versus 5.30 L/h/70 kg, respectively).[24] Estimated oral bioavailability in pediatric patients was greater than that reported previously for immunocompromised pediatric patients and adults with IA (85% versus 64%, respectively).[21,24] The oral bioavailability of voriconazole in healthy adults has been estimated to be greater than 90%.[12,25] The large interindividual variability in oral bioavailability and voriconazole exposure seen in these patients may be because of them receiving many concomitant medications and having various serious underlying conditions, which could affect the oral absorption and disposition processes and could not be easily delineated.

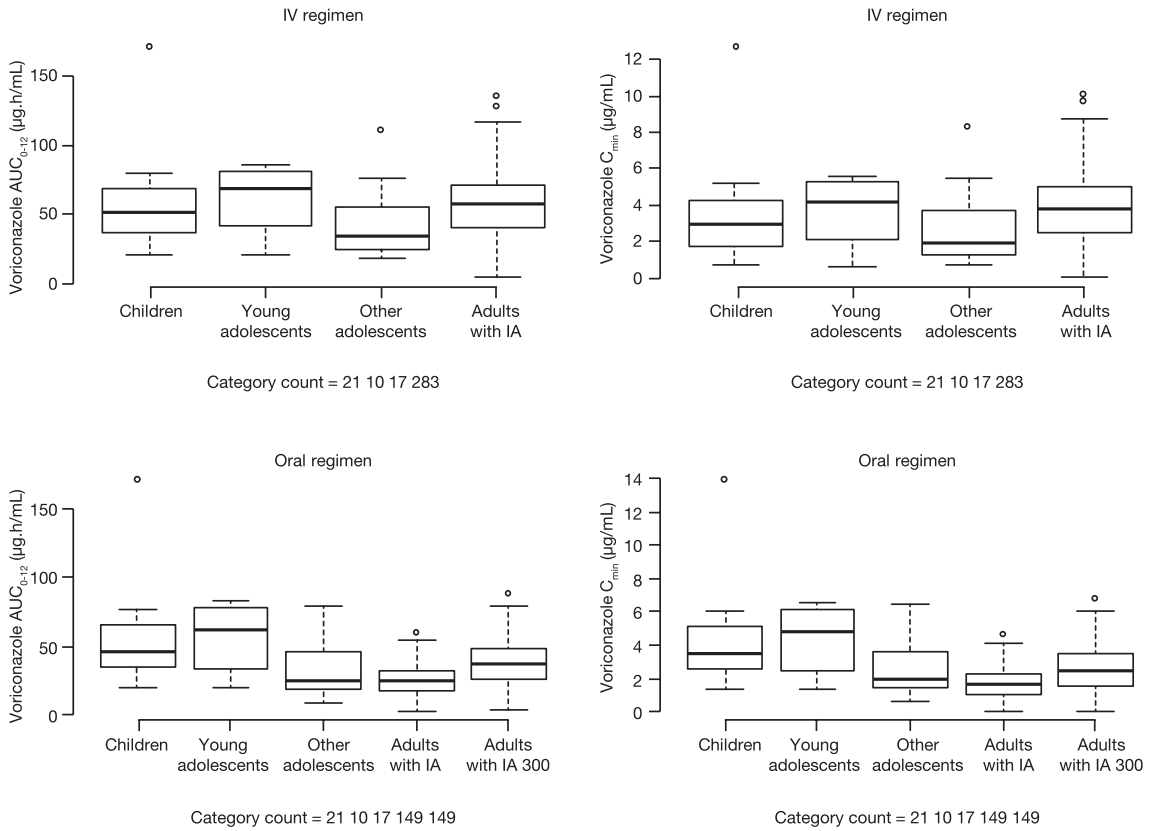

FIGURE 6. Comparison of estimated steady-state voriconazole $AUC_{0–12}$ and C_{min} by age group at matching IV and oral doses in patients with IA. Horizontal center line represents the median; box represents the interquartile distance; whiskers represent ≤1.5 × interquartile range; outliers are represented by open circles beyond whiskers. Intravenous regimen: 8 mg/kg q12 h for children (aged 2–<12 years) and young adolescents (aged 12–14 years weighing <50 kg); 4 mg/kg q12 h for all other adolescents and adults with IA. Oral regimen: 9 mg/kg (max 350 mg) q12 h for children (aged 2–<12 years) and young adolescents (aged 12–14 years weighing <50 kg); 200 mg q12 h for all other adolescents and adults with IA. Note that data from adults with IA at 300 mg oral q12 h are also included here and denoted as "Adults with IA 300". This figure was created for easy comparison across different age groups. $AUC_{0–12}$ indicates area under the curve from 0 to 12 h; C_{min}, minimum plasma concentration (trough); IA, invasive aspergillosis; IV, intravenous; q12 h, every 12 h.

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The current analysis is consistent with previous findings in adults with IA where CYP2C19 genotyping status did not have a clinically relevant effect on voriconazole exposure.[24] A recently published article concluded that a CYP2C19 genotype-directed dosing algorithm (ie, 5, 6 or 7 mg/kg q12 h stratified by CYP2C19 status) allowed pediatric patients (n = 20) to reach target voriconazole concentration significantly sooner than pediatric patients with a standard dosing regimen (5 mg/kg q12 h, n = 25).[26] Of note, the doses evaluated in that publication are lower than those investigated in our studies. It is possible that the use of lower doses in these pediatric patients might be an important factor in the delay of reaching target concentration, in addition to the CYP2C19 polymorphism. CYP2C19 is known to be the major pathway for voriconazole metabolism, but notably other pathways, such as CYP3A4 and CYP2C9, are also involved and consequently CYP2C19 genotype alone does not explain the variability in voriconazole exposure. The impact of genotype on voriconazole exposure can be influenced by a patient's demographic characteristics, underlying disease and concomitant medications. Hence, voriconazole dose adjustment solely based on CYP2C19 genotype is not currently recommended.

Approximately 42% of pediatric patients received omeprazole or esomeprazole (CYP2C19 inhibitors known to increase voriconazole exposure in healthy subjects[27]). Although no trend was identified in our assessment, the impact of these concomitant medications on voriconazole exposure cannot be ruled out. Similarly, approximately 30% of adults with IA for comparison also received concomitant omeprazole or esomeprazole.[24] At matching IV doses, average exposure values and distributions were similar in these pediatric patients and adult patients with IA (Fig. 6). At matching oral doses, average exposures in pediatric patients were greater than that in adult patients with IA; however, substantial overlap in exposure distributions was observed between groups (Fig. 6). Considering that treatment is being provided for potentially life-threatening infections, it is preferred to start with a dose with relatively high exposure to ensure sufficient coverage and then reduce to lower doses if needed.

Although an association between increased voriconazole exposure and hepatic AEs was established (with multiple-panel data only), voriconazole concentrations could not be used to accurately predict hepatic AE occurrence given the large uncertainty of prediction (Fig. 4A and B). Note that the multiple-panel data analysis may have overestimated the AE occurrence probability, as a patient with multiple AEs would be counted several times.

The lack of association of voriconazole exposure with efficacy and other safety end points may be because of an insufficient sample size. These findings are consistent with what has previously been reported in adult patients with IA.[24] These patients typically had multiple comorbidities and received multiple medications. In addition, treatment effect is just one of the contributing factors leading to successful clinical outcomes for life-threatening fungal infections. Patients' underlying conditions and ability to respond to the treatment are also important factors influencing the clinical outcomes. No consensus regarding correlations of voriconazole exposure with clinical outcomes and treatment-related toxicity has been established because of the complexity of fungal infections in the clinical setting, despite substantial efforts to do so.[9,10,28–37] Therefore, the clinical response and tolerability of individual patients should continue to be the primary consideration for dose adjustment, and voriconazole C_{min} (if available) should be considered as a secondary marker for the purpose of dose adjustment.

In our studies, most pediatric patients (64%; n = 34) did not require dose adjustments; 26% (n = 14) had dose reductions and 11% (n = 6) had dose escalations. One patient had dose reduction and dose escalation during the treatment period. Among them, 9 patients had dose reductions because of high voriconazole concentrations, whereas 3 had dose escalations because of low concentrations based on predefined provisional instructions. Dose adjustment with 1 mg/kg (50 mg oral) was sufficient for all but 3 patients with IA, indicating that slight adjustment of the initial dose was generally adequate. Taken together, the proposed dosing regimens were deemed acceptable as the initial recommendation for pediatric patients.

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Invasive aspergillosis in patients with hematological malignancies in the Czech and Slovak republics: Fungal InfectioN Database (FIND) analysis, 2005–2009

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SUMMARY

Objectives: To evaluate risk factors, diagnostic procedures, and treatment outcomes of invasive aspergillosis (IA) in patients with hematological malignancies.
Methods: A retrospective analysis of data from proven/probable IA cases that occurred from 2005 to 2009 at 10 hematology centers was performed.
Results: We identified 176 IA cases that mainly occurred in patients with acute leukemias (58.5%), mostly those on induction/re-induction treatments (39.8%). Prolonged neutropenia was the most frequent risk factor for IA (61.4%). The lungs were the most frequently affected site (93.8%) and computed tomography detected abnormalities in all episodes; however, only 53.7% of patients had findings suggestive of IA. Galactomannan (GM) detection in serum or bronchoalveolar lavage fluid (positive in 79.1% and 78.8% of episodes, respectively) played a crucial role in IA diagnosis. Neutrophil count and antifungal prophylaxis did not influence the GM positivity rate, but empirical therapy decreased this rate (in serum). Of the IA cases, 53.2% responded to initial antifungal therapy. The combination of voriconazole and echinocandin, even as initial or salvage therapy, did not perform better than voriconazole monotherapy ($p = 0.924$ for initial therapy and $p = 0.205$ for salvage therapy). Neutrophil recovery had a significant role in the response to initial (but not salvage) antifungal therapy.
Conclusions: Our retrospective analysis identified key diagnostic and treatment characteristics, and this understanding could improve the management of hematological malignancy patients with IA.
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1. Introduction

Invasive fungal diseases (IFD) are an important cause of morbidity and mortality in patients with hematological diseases.[1,2] The epidemiology of IFD in this group of severely immunocompromised patients has changed substantially during the last two decades, with invasive aspergillosis (IA) being a predominant infection.[1] The incidence of this infection can vary and is mainly based on the underlying hematological malignancy; it can reach up to 10% among patients undergoing treatment for acute leukemia or allogeneic hematopoietic stem cell transplantation (HSCT).[3] However, there have been several key advancements over the past decade that have significantly improved not only the diagnosis (widespread availability of high-resolution computed tomography (HRCT) and non-culture based diagnostic tools, such as the detection of galactomannan (GM)), but also treatment

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options (availability of new antifungal drugs, e.g., voriconazole and echinocandins) of IA. These events have led to the recently reported improvement in the prognosis of patients with this life-threatening infection.[2,4,5] Moreover, several observational registries in Europe, as well as worldwide, have been created with the goal of collecting real world data regarding incidence, risk factors, and treatment outcomes of patients with IA.[1,2,4–6]

In this multicenter study, we report data from IA episodes that occurred in patients with hematological malignancies. These data were retrospectively collected from the Fungal InfectioN Database (FIND), which holds data from almost all hematology centers in the Czech and Slovak republics. The aim of this study was to analyze the risk factors, diagnostic procedures, and treatment outcomes from the largest cohort of IA episodes in Central Europe published to date.

2. Methods

2.1. Design

Thirteen hematology centers in the Czech and Slovak republics participate in the FIND project. The database consists of retrospectively collected data of proven and probable IA cases that occurred between 2001 and 2009, as well as a prospective collection of cases from 2010 onwards.

This study was conducted by performing an analysis of proven and probable IA cases that occurred between January 1, 2005 and December 31, 2009, which had been retrospectively entered as electronic case report forms by 10 of 13 participating centers (seven adult and three pediatric centers). The distribution of episodes during this time period was not uniform and was mainly dependent on the extension of non-culture-based diagnostic techniques (e.g., GM detection) among centers. Therefore, the number of episodes in individual time intervals does not reflect the real incidence of infection. Forty-one percent of cases entered into the database and analyzed occurred between 2005 and 2007, 59% between 2008 and 2009.

2.2. Case identification

Cases were identified in participating centers by reviewing the patient charts as well as laboratory, microbiology, and imaging results. Pathology reports from autopsies were also used. All identified episodes of IA during the observation period were included in the database.

The variables collected in the electronic case report forms included the subject's demographic characteristics, underlying hematological malignancy and treatment, clinical signs and symptoms, and the results of microbiological and histological investigations, as well as results of imaging studies, information regarding the use of mold-active antifungal prophylaxis and empirical antifungal treatment, targeted antifungal treatment and outcomes, neutrophil counts at the time of diagnosis as well as before and after each antifungal treatment, and finally patient survival. Due to the retrospective design of this study, a patient's informed consent was not required. The Institutional Review Board of the University Hospital Brno approved this study.

2.3. Definitions

Episodes of IA were defined according to the 2002 European Organisation for Research and Treatment of Cancer and Mycosis Study Group (EORTC/MSG) criteria.[7] The day of diagnosis was defined as the day when criteria for proven or probable IA were fulfilled. Empirical antifungal therapy was defined as the administration of systemic antifungal treatment in patients with persistent fever only, or in patients who did not fulfill criteria for proven or probable IFD at the time of treatment initiation. Targeted antifungal therapy was started when patients fulfilled criteria for proven or probable IA. The overall outcome of therapy, as well as the outcome of each line of antifungal treatment, was classified according to published EORTC/MSG recommendations.[8] The effect of therapy was evaluated only if the targeted antifungal therapy lasted at least 5 days. An independent, blinded evaluation of all the entered data was performed by a review board at the main study center, with special consideration to the fulfillment of EORTC/MSG criteria for the diagnosis of proven or probable IA, as well as treatment outcome.

2.4. Statistical analysis

Frequency tables and standard descriptive statistics were used for summation of the patient characteristics. Proportions were compared with the maximum-likelihood Chi-square test or Fisher's exact test. Continuous variables were compared with the Mann–Whitney or Kruskal–Wallis analysis of variance

Table 1
Baseline characteristics

Patients	
No. of patients	176
Age, years, median (range)	56 (3–77)
Sex, male/female, *n* (%)	104 (59.1%)/ 72 (40.9%)
Patient's disease at baseline, *n* (%)	
AML + MDS	73 (41.5%)
ALL	30 (17.0%)
NHL + HL	27 (15.3%)
CLL	20 (11.4%)
MM	12 (6.8%)
CML + CMPD	4 (2.3%)
Other	10 (5.7%)
Anticancer therapy during/before IA, *n* (%)	
Induction/reinduction therapy of acute leukemia	70 (39.8%)
Allogeneic HSCT	30 (17.0%)
Autologous HSCT	17 (9.7%)
Other	52 (29.5%)
None	7 (4.0%)
Presence of risk factors for development of IA, *n* (%)	
Neutropenia $<0.5 \times 10^9$/l for >10 days	108 (61.4%)
Administration of corticosteroids for >21 days	50 (28.4%)
Pulmonary/respiratory tract disease in anamnesis (COPD, etc.)	22 (12.5%)
GVHD	20 (11.4%)
Other risk factors	41 (23.3%)
Number of risk factors present at diagnosis, *n* (%)	
0	29 (16.5%)
1	79 (44.9%)
2	44 (25.0%)
≥3	24 (13.6%)
IA episodes	
No. of episodes	176
Certainty of diagnosis according to EORTC/MSG 2002 criteria, *n* (%)	
Proven IA	27 (15.3%)
Probable IA	149 (84.7%)
Site of infection, *n* (%)	
Lung	165 (93.8%)
Sinuses	1 (0.6%)
Disseminated	7 (4.0%)
Other	3 (1.7%)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CMPD, chronic myeloproliferative disease; COPD, chronic obstructive pulmonary disease; EORTC/MSG, European Organisation for Research and Treatment of Cancer/Mycoses Study Group; GVHD, graft-versus-host disease; HL, Hodgkin lymphoma; HSCT, hematopoietic stem cell transplantation; IA, invasive aspergillosis; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin lymphoma.

(ANOVA) test, as appropriate. The probabilities of overall survival were estimated using the Kaplan–Meier method, and a comparison of survival in the groups of patients was performed using a log-rank test. The point estimates were supplied with 95% confidence intervals (CI). A level of statistical significance $\alpha = 0.05$ was used in all analyses. For the analysis of the role of neutrophil count at the defined time points in the efficacy of antifungal treatment, patients were divided into three groups: those with a neutrophil count $\leq$0.1, 0.1–1.0, and $\geq 1.0 \times 10^9$/l. Analyses were performed using statistical software SPSS 12.0.2 for Windows (SPSS Inc., 2003) and STATISTICA 9.0.1 for Windows (StatSoft, Inc. 2010).

3. Results

3.1. Characteristics of patients and episodes of IA

During the study period (2005–2009), 176 episodes of IA occurring in 176 patients were identified: 27 (15.3%) proven and 149 (84.7%) probable. Patient characteristics are shown in Table 1. Acute leukemias represented the majority of the underlying hematological diseases (58.5%), and induction or re-induction treatment for acute leukemia (but not allogeneic HSCT) represented the most frequent anticancer treatment (39.8%). Therefore, patients with active acute leukemia during the first induction or salvage therapy represented the typical population of hematological malignancy patients with the highest risk of IA. Based on these data, it is not surprising that the most common classical risk factor identified in 61.4% of IA episodes was profound and prolonged neutropenia (Table 1). The lung was the most commonly affected site (93.8%), with 21 (12.0%) proven and 144 (81.8%) probable episodes. In addition, disseminated and isolated extrapulmonary infections were rare (4.0% and 2.3%, respectively).

3.2. Signs of infection

Out of the 176 patients with IA, 136 (77.3%) had fever at the time of diagnosis, with a median duration of 6 days before diagnosis (range 0–53 days before diagnosis; interquartile range (IQR) 3–11 days before diagnosis). Moreover, 54.0% of patients with IA fulfilled criteria for persistent fever despite the administration of broad-spectrum antibiotics for 5 days. Out of 165 patients with invasive pulmonary aspergillosis (IPA), 125 (75.8%) exhibited at least one sign that was suggestive of pulmonary disease, which developed within a median of 5 days before diagnosis (range 0–35 days; IQR 2–9 days). The spectrum of these signs is shown in Table 2.

3.3. Diagnostic procedures

3.3.1. Imaging studies

A chest X-ray was performed at the time of diagnosis in 152/165 (92.1%) patients with IPA. However, abnormalities were only identified in 73.0% of those patients. Moreover, the most commonly observed abnormality was a non-specific infiltrate (44.7%) (Table 2). In contrast, chest HRCT, which was performed

Table 2
Clinical manifestations and results of diagnostic tests at the time of diagnosis of invasive aspergillosis

Clinical manifestations at the time of diagnosis, all patients (N = 176), n (%)	
Fever >38.0 °C	136 (77.3%)
Fever not responding to 5 days of antibiotics	95 (54.0%)
Presence of organ-specific clinical symptoms	134 (76.1%)
Clinical signs in patients with IPA (n = 165), n (%)	
Any symptom	125 (75.8%)
Cough	69 (41.8%)
Dyspnea	37 (22.4%)
Chest pain	11 (6.7%)
Hemoptysis	2 (1.2%)
Other	6 (3.6%)
Chest X-ray abnormality in patients with IPA (n = 152),[a] n (%)	
Any abnormality	111 (73.0%)
Non-specific infiltrate(s)	68 (44.7%)
Nodule(s)	36 (23.7%)
Interstitial process	1 (0.7%)
Pleural effusion	1 (0.7%)
Cavitation(s)	1 (0.7%)
Other	4 (2.6%)
Chest high-resolution CT abnormality in patients with IPA (n = 149),[a] n (%)	
Any abnormality	149 (100%)
Predominant abnormality	
Non-specific infiltrate(s)	69 (46.3%)
Halo sign	40 (26.8%)
Macronodule(s) >1 cm	17 (11.4%)
Cavitation	9 (6.0%)
Micronodule(s) <1 cm	8 (5.4%)
Pleural effusion	5 (3.4%)
Air crescent sign	1 (0.7%)
Laboratory test results at the time of diagnosis, all patients, n (%)	
Serum galactomannan positive (consecutive index of positivity >0.5) (n = 172)[a]	136 (79.1%)
Serum (1→3)-β-D-glucan positive (single value >80 pg/ml) (n = 44)[a]	36 (81.8%)
Mycological examination, microscopy positive (all materials) (n = 71)[a]	9 (12.7%)
Mycological examination, culture positive (all materials) (n = 81)[a]	24 (29.6%)
Histology positive (all materials) (n = 12)[a]	8 (66.7%)
BAL fluid examination in patients with IPA, n (%)	
Mycological examination, microscopy positive (n = 49)[a]	5 (10.2%)
Mycological examination, culture positive (n = 48)[a]	9 (18.8%)
BAL fluid galactomannan positive (index of positivity >0.5) (n = 66)[a]	52 (78.8%)

BAL, bronchoalveolar lavage; CT, computed tomography; IPA, invasive pulmonary aspergillosis.
[a] Calculated only for patients for whom the test was performed.

in 149/165 (90.3%) patients with IPA at the time of diagnosis (2005–2007, 87.8% vs. 2008–2009, 90.2%, $p = 0.620$), detected an abnormality in all of these patients. Interestingly, the most frequently observed abnormality on these early HRCT scans was a non-specific infiltrate (46.3%). Signs that are more specific for IFD, such as a halo sign, nodules, or cavitations, were seen substantially less frequently (Table 2). There was no statistically significant difference in the frequency of individual abnormalities on HRCT scans between patients with neutropenia (neutrophils $<1.0 \times 10^9$/l) and those without ($p = 0.378$).

3.3.2. Non-culture diagnostic techniques—serum

The GM test was performed at all centers for screening (2–3 times per week) in high-risk patients (e.g., patients receiving induction for acute leukemia or undergoing allogeneic HSCT) and on request in all other patients with abnormalities on imaging studies. GM assessment of at least two serum samples was performed in 172/176 (97.7%) patients with IA (2005–2007, 95.9% vs. 2008–2009, 99.0%, $p = 0.176$). Using the criterion of an index of positivity >0.5 from two consecutive serum samples as a positive test result, we found the test positive in 79.1% of tested episodes (Table 2), and a positive result of the GM assay (consecutive positivity) preceded the final diagnosis of IA by a median of 2 days (range 0–34 days; IQR 1–4 days). The rate of positive test results was not influenced by the neutrophil count at the time of diagnosis ($p = 0.426$) or by the administration of mold-active antifungal prophylaxis ($p = 0.854$). In contrast, empirical antifungal therapy using a mold-active antifungal drug at the time of diagnosis of IA significantly decreased the proportion of positive GM test results in serum compared to patients not receiving the treatment (67% vs. 88%, respectively; $p = 0.001$). The median serum GM index of positivity level at the time of IA diagnosis was 1.28 (range 0.11–11.46). The detection of 1→3-β-D-glucan (BG) was available at only one center, and therefore the test was performed in only 44/176 (25.0%) patients. A positive test result (BG concentration >80 pg/ml from a single serum sample as the cut-off) was recorded in 81.8% of these patients (Table 2).

3.3.3. Mycological examination

Histological examination, microscopic evaluation, and cultures of any relevant clinical specimens were performed in 12/176 (6.8%), 71/176 (40.3%), and 81/176 (46.0%) patients with IA, respectively. However, with the exception of the histological examination, which was positive in 66.7% of a very limited number of samples obtained by biopsy, the rate of positive results of the other two conventional techniques was very low (12.7% and 29.6%, respectively) (Table 2).

Aspergillus fumigatus represented 19/24 (79.2%) identified isolates, followed by *Aspergillus flavus* 1/24 (4.2%), *Aspergillus niger* 1/24 (4.2%), *Aspergillus terreus* 1/24 (4.2%), and other *Aspergillus* species 2/24 (8.3%).

3.3.4. Bronchoalveolar lavage (BAL) fluid analysis

Since IPA predominated in our patient group, BAL fluid was the most frequent mycologically evaluated material (Table 2). However, conventional mycological techniques with a very low frequency of positive results (10.2% microscopy and 18.8% culture) did not contribute substantially to the diagnosis of IPA in this group of patients. In contrast, the GM assay was positive in 52 out of 66 (78.8%) obtained BAL fluids using a cut-off value of 0.5. The rate of GM assay positivity in BAL fluid was not influenced by neutrophil count ($p = 0.580$) or the administration of mold-active antifungal prophylaxis ($p = 0.147$), and in contrast to serum was not influenced by empirical antifungal therapy (76% vs. 81%; $p = 0.607$).

3.4. Prophylaxis and empirical treatment

Of the 176 patients with IA, 44 (25.0%) had received mold-active antifungal prophylaxis, with a median treatment time of 24 days (range 4–227 days; IQR 16–42 days) (Table 3). More than half of these episodes developed under prophylaxis treatment with itraconazole (25/44, 56.8%); however, itraconazole was also the most frequently used anti-mold prophylaxis at the time our study was performed. Moreover, the azole plasma concentration before breakthrough infection was only available in two patients.

At the time of diagnosis of IA, 76/176 (43.2%) patients had already received mold-active empirical antifungal treatment, and the most frequently used was conventional amphotericin B (30.3% of empirically treated patients) (Table 3). The length of empirical treatment before the definitive diagnosis of IA was short (median 6 days, range 2–44 days, IQR 4–11 days). Therefore, this relatively high number of empirically treated patients reflects the suspicions of the clinician to IFD and early administration of systemic antifungals, rather than a high number of breakthrough IFD cases during prolonged antifungal therapy.

3.5. Antifungal therapy

Targeted antifungal therapy for proven and probable IA was administered in 156/176 (88.6%) patients. In addition, 71 (40.3%) patients received only one line of therapy, 61 (34.7%) patients received treatment with a second-line therapy for toxicity or failure of the previous therapy, and 24 (13.6%) patients received more than two lines of antifungal therapy. Neither the spectrum of antifungal drugs nor their combinations used for the treatment of IA differed between the two observed periods ($p = 0.252$, $p = 0.229$, and $p = 0.622$, for first line, second line, and further lines, respectively).

A complete or partial response to treatment was achieved in 83/156 (53.2%) patients treated with first-line therapy (median length of first-line therapy 15 days, range 5–139 days, IQR 10–25 days). There was no substantial difference in the response rate between the two most frequently used approaches: voriconazole monotherapy and a combination of voriconazole and echinocandin (61.9% vs. 61.0%, respectively; $p = 0.924$) (Table 4). Forty (25.6%) of the 156 patients treated with first-line therapy received salvage therapy for failure of this treatment (median duration 19 days, range 5–159 days, IQR 10–32 days). Although the number of these patients was limited, the combination of voriconazole and echinocandin did not provide a better therapeutic outcome in this setting compared to voriconazole monotherapy ($p = 0.205$) (Table 4).

Table 3
Antifungal prophylaxis and empirical antifungal therapy

Anti-mold prophylaxis at the time of IA diagnosis	
Present	44 (25.0%)
Antifungal drug used[a]	
Itraconazole	25 (56.8%)
Voriconazole	7 (15.9%)
Posaconazole	6 (13.6%)
Conventional amphotericin B	4 (9.1%)
Echinocandin	2 (4.5%)
Anti-mold empirical antifungal therapy at the time of IA diagnosis	
Present	76 (43.2%)
Antifungal drug used[a]	
Conventional amphotericin B	23 (30.3%)
Lipid formulation of amphotericin B	20 (26.3%)
Voriconazole	13 (17.1%)
Echinocandin	12 (15.8%)
Other	9 (11.8%)

IA, invasive aspergillosis.
[a] Percentage calculated from patients receiving treatment.

Table 4
Targeted antifungal therapy—efficacy of first-line and salvage therapy

	n	Treatment response			
		Complete or partial response	Stable disease	Progression	Not known
First-line therapy	156	83 (53.2%)	20 (12.8%)	53 (34.0%)	-
Voriconazole	63	39 (61.9%)	8 (12.7%)	16 (25.4%)	-
Combination of echinocandin + voriconazole	41	25 (61.0%)	3 (7.3%)	13 (31.7%)	-
Conventional AMB	13	4 (30.8%)	3 (23.1%)	6 (46.2%)	-
Lipid formulation of AMB	13	7 (53.8%)	1 (7.7%)	5 (38.5%)	-
Echinocandin	9	2 (22.2%)	-	7 (77.8%)	-
Other	17	6 (35.3%)	5 (29.4%)	6 (35.3%)	-
Salvage therapy	40	15 (37.5%)	7 (17.5%)	17 (42.5%)	1 (2.5%)
Voriconazole	9	2 (22.2%)	3 (33.3%)	3 (33.3%)	1 (11.1%)
Combination of echinocandin + voriconazole	7	4 (57.1%)	2 (28.6%)	1 (14.3%)	-
Lipid formulation of AMB	3	1 (33.3%)	1 (33.3%)	1 (33.3%)	-
Other	21	8 (38.1%)	1 (4.8%)	12 (57.1%)	-

AMB, amphotericin B.

To shorten the period of neutropenia, 97/176 (55.1%) patients received granulocyte colony stimulating factors. Granulocyte transfusions were not used. Of the 176 patients, 10 (5.7%) underwent surgery in addition to chemotherapy.

At the end of all targeted treatment approaches efficacy was evaluated. The median length of treatment was 32.5 days (range 5–148 days, IQR 17–66 days), and 105 out of 156 (67.3%) patients responded; however, 50/156 (32.1%) patients failed and one patient was not evaluable. Secondary prophylaxis (mostly with voriconazole) was used in 71/176 (40.3%) patients with a median length of treatment of 48 days (range 10–512 days; IQR 21–78 days).

3.6. The role of neutrophils in the efficacy of antifungal treatment

There was no statistically significant difference in the percentage of patients with a successful treatment outcome (complete and partial response) at the end of all antifungal therapies based on neutrophil count at the start of antifungal treatment ($p = 0.423$). This lack of difference was also found when the role of neutrophils at the start of the first treatment and salvage therapy and the treatment outcome at the end of these therapies was evaluated separately (Table 5).

In contrast, there was a statistically significant increase in the percentage of patients who successfully responded (complete and partial response) at the end of all antifungal therapies with increasing neutrophil counts at the end of antifungal treatment ($p < 0.001$) (Table 5). A substantially higher response rate was identified in patients with neutrophil counts $>1.0 \times 10^9$/l at the end of the first-line treatment compared to patients with neutrophil counts of 0.1–1.0 ($p = 0.007$) and <0.1 ($p < 0.001$) $\times 10^9$/l. However, we did not find a role of neutrophil counts at the end of salvage therapy in patients receiving this treatment ($p = 0.432$) (Table 5).

Finally, the change in neutrophil count during IA therapy and treatment outcome was analyzed. During first-line treatment, patients with a successful treatment outcome (complete and partial response of IA) had a significant increase in neutrophil count ($p < 0.001$ and $p = 0.003$, respectively). Moreover, the median neutrophil count in patients with a complete or partial response increased during the treatment from neutropenic range ($<1.0 \times 10^9$/l) to non-neutropenic range (Figure 1A). In contrast, patients with treatment failure were persistently neutropenic (progression of IA) or did not reveal any significant increase in their neutrophil count during therapy (stable IA) (Figure 1A). A similar analysis was performed for patients receiving salvage therapy, and no significant increase in neutrophil count was observed in any treatment outcome group (Figure 1B); however, the number of patients was limited.

3.7. Survival

The median survival in our patient group was 28.1 (95% CI 15.6–40.7) weeks. The 3- and 12-month overall survival (OS) was 57.8% (95% CI 50.5–65.1%) and 43.0% (95% CI 35.4–50.5%), respectively. OS follows survival attributed to IA (OS_{IA}), thus IA was the predominant cause of death during the first 3 months after diagnosis, while other causes (mainly underlying diseases) were

Table 5
The role of neutrophil count at the start and at the end of antifungal therapy in treatment outcome

	Patients with successful treatment outcome (complete or partial response) at the end of therapy (%)[a]			
	Neutrophils $<0.1 \times 10^9$/l	Neutrophils $0.1–1.0 \times 10^9$/l	Neutrophils $>1.0 \times 10^9$/l	*p*-Value[b]
Neutrophil count at the start of:				
Any therapy ($n = 143$)	63.8%	76.7%	70.5%	0.423
First-line therapy ($n = 144$)	50.7%	53.3%	64.4%	0.341
Salvage therapy[c] ($n = 30$)	37.5%	66.7%	25.0%	0.195
Neutrophil count at the end of:				
All therapies ($n = 128$)	21.1%	50.0%	80.9%	<0.001
First-line therapy ($n = 129$)	16.7%	35.0%	68.2%	<0.001
Salvage therapy[c] ($n = 32$)	20.0%	57.1%	40.0%	0.432

[a] The treatment outcome was evaluated at the end of therapy given in the raw (i.e., the end of all received therapies, the end of first-line therapy, or the end of salvage therapy, respectively).
[b] Maximum-likelihood Chi-square test, difference between all three groups according to neutrophil count.
[c] Salvage was defined as treatment after failure of first-line therapy.

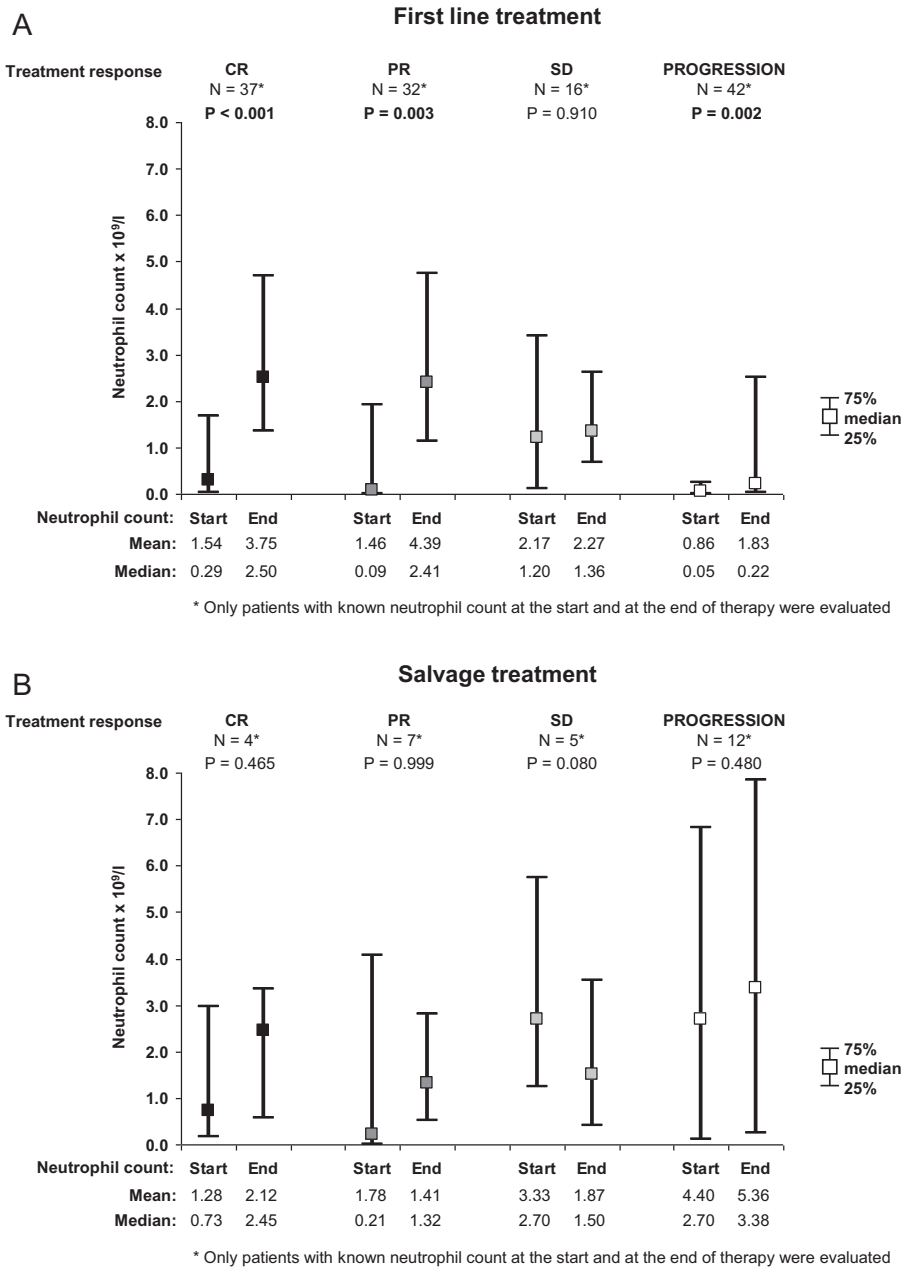


Figure 1. Change in neutrophil count during (A) first-line and (B) salvage therapy of invasive aspergillosis (CR, complete response; PR, partial response; SD, stable disease).

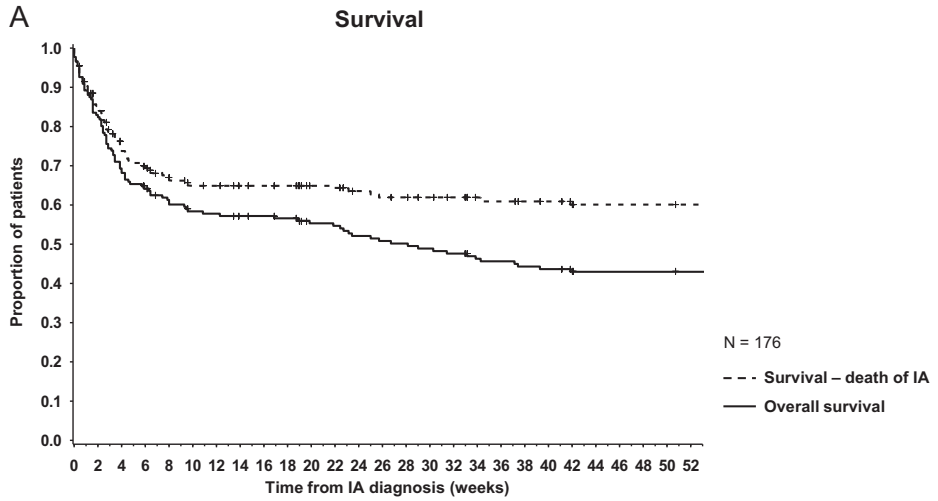
predominantly responsible for death in patients who survived longer than 3 months (Figure 2A). Patients with probable IA had significantly better OS as well as survival attributed to IA (OS_{IA}) (Figure 2B). OS as well as OS_{IA} did not differ between cases diagnosed during 2005–2007 compared to more recent episodes (2008–2009) (OS: $p = 0.173$, 63.4% (95% CI 52.4–74.4%) vs. 52.7% (95% CI 42.9–62.4%) at 3 months, respectively; OS_{IA}: $p = 0.366$, 70.7% (95% CI 60.1–81.3%) vs. 60.8% (95% CI 51.0–70.6%) at 3 months, respectively).

4. Discussion

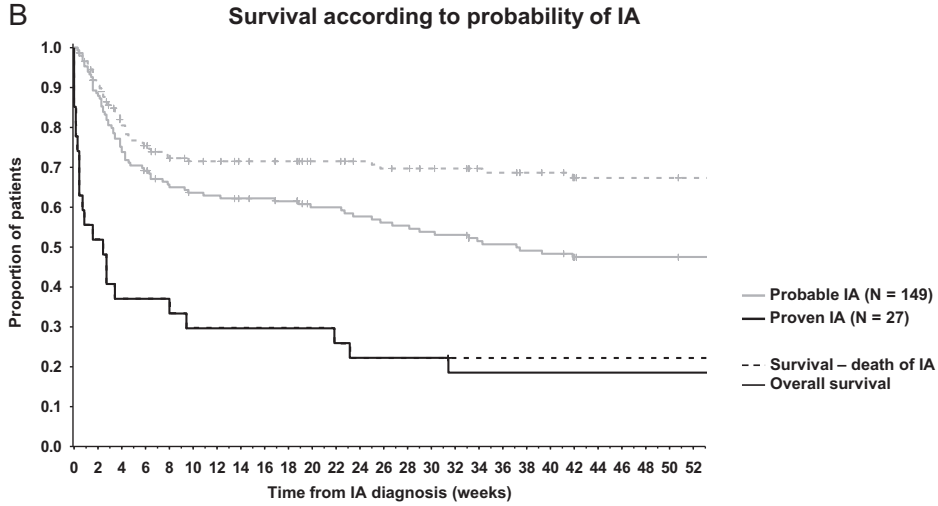
This is the largest multicenter study published to date that has analyzed episodes of IA in hematological malignancy patients from Central Europe. FIND is a network of hematology centers that gather and share information to improve our understanding of epidemiology, diagnostics, therapy, and the outcome of IFD in hematological malignancy patients from the Czech and Slovak republics.

Our analysis confirmed several published and generally accepted facts in the view of risk factors, diagnostics, and treatment of this infection among patients with hematological malignancies.[2,4,6,9–16]

The significance of our study clearly lies in several unique findings, which should be noted. First, although we have shown the importance of using early lung HRCT for the diagnosis of pulmonary abnormalities (all patients with IPA had some detectable abnormality), only 53.7% had findings that were described as 'specific' for invasive mold infection based on EORTC/MSG 2008 criteria.[17] Therefore, half of our IPA patients had non-specific infiltrates on early HRCT scans, of which the performance was generally driven by persistent fever or GM results. Recent studies have shown that the neutrophil count plays a role in the pattern of findings on imaging studies.[11,18] However,



Weeks from IA diagnosis	Overall survival (%)	Survival – death of IA (%)
2	82.4 (76.8; 88.0)	84.5 (79.2; 89.9)
6	64.2 (57.1; 71.3)	69.5 (62.5; 76.4)
12	57.8 (50.5; 65.1)	65.0 (57.7; 72.2)
30	48.9 (41.4; 56.4)	61.9 (54.4; 69.4)
52	43.0 (35.4; 50.5)	60.1 (52.4; 67.8)



Weeks from IA diagnosis	Overall survival (%) p < 0.001		Survival – death of IA (%) p < 0.001	
	Probable IA	Proven IA	Probable IA	Proven IA
2	87.9 (82.7; 93.2)	51.9 (33.0; 70.7)	90.5 (85.7; 95.2)	51.9 (33.0; 70.7)
6	69.1 (61.7; 76.5)	37.0 (18.8; 55.2)	75.4 (68.3; 82.5)	37.0 (18.8; 55.2)
12	62.9 (55.1; 70.7)	29.6 (12.4; 46.9)	71.6 (64.1; 79.1)	29.6 (12.4; 46.9)
30	53.8 (45.6; 62.0)	22.2 (6.5; 37.9)	69.7 (61.9; 77.4)	22.2 (6.5; 37.9)
52	47.5 (39.2; 55.8)	18.5 (3.9; 33.2)	67.5 (59.4; 75.5)	22.2 (6.5; 37.9)

Figure 2. Overall survival and survival attributed to invasive aspergillosis (IA) for (A) all patients and (B) based on the probability of IA diagnosis.

this does not explain our results. In the study by Nicolle et al.,[4] the patient population and percentage of patients with prolonged neutropenia was similar to our observations in this analysis. However, the authors of that study found a 'halo sign' in 81% of the patients, whereas only 26.8% of patients had the sign in our study. On the other hand, a recent study by Lortholary et al.[6] examining a mixed patient population with 77.6% of patients suffering from a hematological malignancy found nodules in the majority of patients (81.3%) with IPA. However, nodules were again rarely found in our study (16.8%). Moreover, we did not observe any significant difference in the frequency of individual abnormalities, including the frequency of non-specific infiltrates in patients with and without neutropenia. Therefore, despite the multicenter approach whereby CT evaluations were performed by local radiologists, one of the explanations for the significant proportion of non-specific findings could be the promptitude of HRCT usage in patients with persistent fever or GM positivity, which has been seen in the last few years due to better availability of this technique. The median time from an HRCT scan to diagnosis of IA in our study was 0 days. Thus, in daily clinical practice where early CT scans are commonly performed and non-specific infiltrates are more frequently seen, mycological examination of these non-specific lesions for a differential diagnosis becomes very important. This finding was very recently supported by others.[19,20]

Our study also demonstrated the essential role of GM testing for the diagnosis of IA.[21] Since the vast majority of cases represented probable IA and the sensitivity of culture and/or cytology was very limited, the diagnosis of probable IA was typically made using a combination of pulmonary abnormalities on lung HRCT and positivity of a GM assay with serum and/or BAL fluid. In addition, the high rate of positive results of the GM assay in serum (79.1%) and BAL fluid (78.8%) was similar or higher than in a recently published series of hematological patients.[4,6,22]

This multicenter study also found that the routine use of regular and frequent (2–3/week) GM screening is widely used at all hematology centers in both countries and seems common in the countries of Europe,[4,6] but is less frequent or limited in others countries,[9] including the USA.[5] Therefore, GM screening was often used in place of invasive procedures for the differential diagnosis of pulmonary infiltrates. In a study by Perkhofer et al.[9] conducted in Austria, 34% of the patients with invasive mold infections had a biopsy performed, whereas only 9.6% of the patients in our study required a biopsy for final diagnosis. The authors of that study recommend performing biopsies in these patients due to the high frequency of invasive zygomycosis. However, the high rate of positive results of the GM assay (serum and/or BAL) in our study could limit biopsies to only GM-negative infiltrates that are very likely to be of IFD origin. Another reason for performing a biopsy given by Perkhofer et al. is the requirement for culture verification of the infection due to the high frequency of *A. terreus* cases; *A. terreus* is resistant to amphotericin B.[9,23] However, in our study, *A. fumigatus* was still the predominant species, and non-fumigatus *Aspergillus* species were very rare, with *A. terreus* isolated in only one case from our large multicenter series. Finally, the importance of GM detection for the diagnosis of IA in daily clinical practice was demonstrated based on the investigator's questionnaire, which is part of our database (data not shown). In 60.2% of IA episodes, investigators subjectively marked the GM assay result as the criterion on which the IA diagnosis was mainly based, followed by HRCT in 18.8% of episodes and histology in 10.2% of episodes. However, when discussing GM assay results, the possible limitation of the test (extensively reviewed in the last European Conference on Infections in Leukemia (ECIL-3) recommendations[24]) given by the risk of lower sensitivity (e.g., caused by administration of mold-active antifungal drugs) or by false-positive results must always be taken into account.

Regardless of recently published and generally accepted guidelines,[25] 26.3% of patients with IA in our database received a combination antifungal treatment, which was mainly a combination of voriconazole and echinocandin, as an initial therapy of IA. This finding, which has also been reported in other registries,[9,26] reflects the real-life situation, where the treating physician intends to maximize the efficacy of antifungal treatment in this group of highly immunocompromised and frequently critically ill patients, not only at the time when the initial treatment fails, but ideally at the start of therapy. However, regardless of promising results from in vitro[27,28] and animal studies,[29] there is limited evidence for such an approach in the salvage setting,[30,31] and more in the initial treatment[32,33] of IA in the literature. Although our study was retrospective and not randomized, we did not find any difference in the efficacy of voriconazole monotherapy compared to the combination of this azole with echinocandin when used as an initial or salvage therapy. The number of patients with neutropenia ($<1.0 \times 10^9$/l) and the length of therapy were not different between treatment groups. However, we did not collect information about performance status, and therefore we cannot exclude the possibility that patients with a severe clinical condition did not preferentially receive a combination therapy, at least during the initial treatment. Therefore, in order to finally resolve this issue, we should await the results of randomized studies comparing both of these approaches that are currently being conducted.

Finally, even with the availability of new antifungal therapies, a large number of patients still fail. Therefore, the actual immunodeficiency status of each patient will play a crucial role in the treatment outcome. Although neutropenia was the most frequent risk factor found for the development of IA, the neutrophil level, in addition to the antifungal therapy used for treatment, would have an impact on patient prognosis.[34] Cordonnier et al. found no impact of neutropenia on patient prognosis at the time of IA diagnosis.[35] Similarly, in our analysis we did not find any significant role of neutrophil count at the start of antifungal therapy on the efficacy of antifungal treatment (primary as well as salvage). However, similar to data presented by Pagano et al.,[2] which showed that acute myeloid leukemia patients with IA had a higher response rate when they had neutropenic recovery, we found a statistically significant increase in the response rate when the neutrophil count measured at the end of antifungal therapy had increased, regardless of the antifungal drug used for treatment. However, our sub-analysis found this crucial role of neutrophil count at the end of treatment was significant for primary therapy, but was not significant for salvage treatment, which was most likely due to the limited number of patients undergoing salvage therapy. An increase in neutrophil count greater than 1.0×10^9/l during initial therapy was related to a complete and partial response, while patients with progression remained neutropenic. However, we found that the outcome of therapy in patients receiving a second-line treatment may be dependent on factors other than the development of neutrophil count during or at the end of therapy, such as the presence of graft-versus-host disease, persistent corticosteroid use, or hepatic insufficiency.[34]

In conclusion, IA is a life-threatening condition and the most frequent IFD in patients with hematological malignancies that requires rapid and specific diagnostics. Lung HRCT with high sensitivity allows for the detection of pulmonary abnormalities; however, these scans are often very non-specific. Therefore, the combination of HRCT with routine and regular screening of GM in serum and/or BAL fluid provides a better differential and rapid diagnosis of IA in this group of immunocompromised patients. While we do not have data that clearly support the benefit of combination antifungal treatment, we have clearly shown that the development of neutrophil count during IA treatment will be a key factor that will determine the treatment response regardless of the antifungal drug or strategy used.

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SUCCESSFUL TREATMENT OF ASPERGILLUS BRAIN ABSCESS IN A CHILD WITH ACUTE LYMPHOBLASTIC LEUKEMIA AND LIVER FAILURE

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□ *Invasive fungal infection continues to pose a significant threat to immunocompromised patients, with cerebral aspergillosis being among the most feared ones. The authors describe an adolescent girl with acute lymphoblastic leukemia (ALL) with subsequent acute liver failure, who developed*

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an aspergillus brain abscess. The patient was treated with combined antifungal therapy using amphotericin B local instillation, prolonged systemic amphotericin B colloidal dispersion along with vinca alkaloids-containing chemotherapy, followed by neurosurgical débridement and oral voriconazole in the setting of ongoing antileukemic maintenance chemotherapy. Her ALL remains now in complete remission 30 months from diagnosis, with no evidence of fungal infection.

Keywords acute leukemia, CNS aspergillosis, liver failure, treatment

Invasive aspergillosis remains an increasingly common complication in severely immunocompromised patients [1, 2]. Mortality rate for patients with CNS involvement reaches over 90% despite multimodal therapy [3, 4]. As a result, only a few sporadic cases have been reported in the literature. Even though recent reports suggest improved outcome for patients treated with voriconazole [5], optimal treatment for invasive aspergillosis remains elusive [6] and pediatric dosing of voriconazole is still being worked out [7].

Here we report on a girl who experienced successful eradication of an aspergillus brain abscess following protracted combined antifungal therapy and MRI-guided and open neurosurgical interventions in the setting of ongoing intensive antileukemic chemotherapy.

CASE REPORT

A normally developing, charming girl, attending senior high school, was known since age 12 years to have slightly elevated total bilirubin (up to 40 μmol/L) and borderline transaminases with no clinical symptoms. No special investigations had been performed for that condition. Her other history was uneventful. Being 16 years old, she had received hepatitis B vaccination. One week following her second Engerix B dose she was diagnosed with B precursor cell acute lymphoblastic leukemia (ALL), with total bilirubin 125 μmol/L, ALT 16 μkat/L, AST 13.7 μkat/L. Her cerebrospinal fluid was clear of any blasts or signs of inflammation. Molecular and cytogenetic analysis identified complex chromosomal changes as adverse prognostic factor [8].

She was put on treatment according to I-BFM ALL 2002 protocol, which is modified original ALL BFM protocol [9]. The patient experienced rapid blast cell clearance, reaching hematological complete remission (CR) on day 15, with significant drop of bilirubin and ALT levels to less than 1/3 of initial values. While in hematological CR, she started therapy with recombinant erythropoietin beta. After day 25 of the protocol, her condition began to deteriorate again. Total bilirubin rose to 528 μmol/L, conjugated 488 μmol/L, and chemotherapy had to be interrupted on day 30. The radionuclide hepatobiliary iminodiacetic acid (HIDA) scan revealed primary liver impairment, with functional loss of 70%. She was put on ICU and complex supportive therapy (including the maximum allowable dose of *N*-acetylcysteine—300 mg/kg/day) led to gradual improved of her condition,

allowing chemotherapy to be continued after 12 days. At the end of induction therapy she developed second episode of febrile neutropenia, with severe mucositis, and striking, painful severe peripheral neuropathy (both mucositis and neuropathia National Cancer Institute–Common Toxicity Criteria grade 4). No infectious agent was identified in blood and surveillance cultures. Persistent neutropenic fever despite 72 h of standard empirical antibiotic therapy called for addition of conventional amphotericin B, at the dose 0.8 mg/kg/day. Within 2 days on conventional amphotericin B therapy there was a drop in her CRP levels, and the pattern of fever was improved with less common fever spikes.

At this time we observe barefaced, gross, frontal behavior, followed by qualitative and quantitative changes in her consciousness. CT and MRI revealed a space-occupying lesion in her frontal lobes, crossing the midline, 44 × 36 × 38 mm, reaching lateral ventricles, with postcontrast ring enhancement. CSF examination did not help in differential diagnosis. Empirical therapy with amphotericin B colloidal dispersion (ABCD) was initiated at a dose of 6 mg/kg/day [9]. Neurosurgeons considered primary open surgery to be too risky, so following 12 days of such therapy, an MRI-guided stereotactic biopsy was carried out. Histopatology with Gimori methamine silver (GMS) stain showed septated hyphae with sharp angles, consistent with *Aspergillus* spp. (Figures 1, 2). The dose of ABCD was increased to 10 mg/kg/day and local instillation of cAmpho B intracavitary, 10 mg/dose q8h, for 10 days was added. Local treatment was well tolerated, with no serious or attributable adverse events. Liver biopsy was performed during the same procedure and

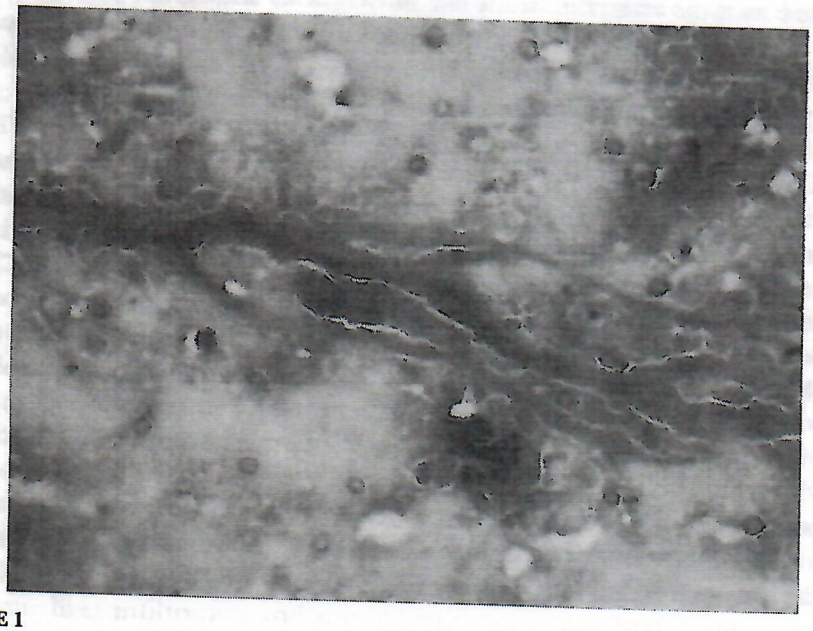

FIGURE 1

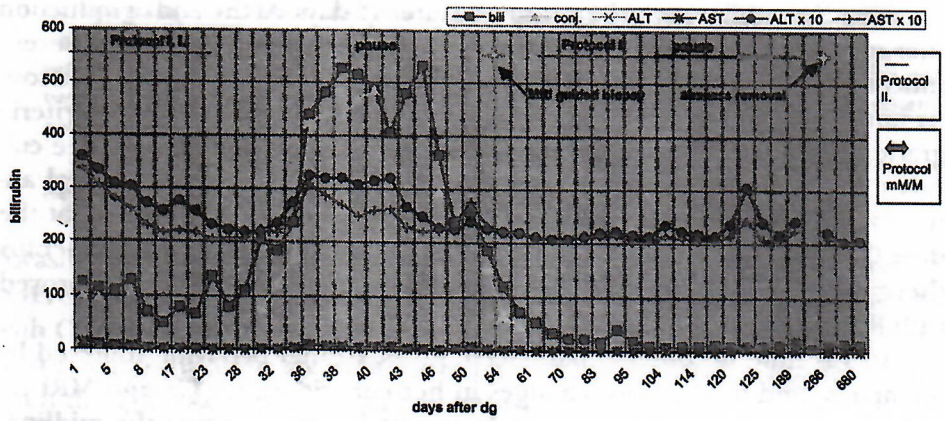

FIGURE 2 Bilirubin, ALT and AST time profiles during treatment course.

revealed chronic, active steatohepatitis, with no evidence of fungal infection and did not help to identify the cause of liver failure. An attempt to add voriconazole to the treatment scheme led to significant drop of renal clearance and rise of ALT, AST, and bilirubin levels, with return to baseline levels after voriconazole withdrawal. Thus, ABCD was continued daily until day 100 and then on every other day for almost 1 year, with daily administration during febrile neutropenia episodes. Chemotherapy was restarted after 42 days of delay during induction. Serial brain MRI showed significant healing and continuing regression of the abscess cavity, despite ongoing intensive chemotherapy courses.

Nine months after the brain lesion diagnosis she underwent successful open neurosurgical removal of the residual lesion. Histhopathology confirmed chronic mycotic abscess: fragmented and partially lysed septated hyphae, with sharp angles, were present centrally, surrounded by demarcating granulomatous resorptive reaction. No fruiting bodies were present. There was gliosis and macrophages filled with debris at the periphery. Because of the fragmentation and lysis of the hyphae, absence of fruiting bodies, and the intensity of the resorptive reaction, the overall downward trend in the biokinetics of the mycosis was obvious. Antimycotic treatment with ABCD was discontinued 3 months after open neurosurgery. During maintenance oral chemotherapy patient developed 3 episodes of febrile neutropenia (fever of unknown origin), where only the empirical addition of voriconazole has led to defervescence of fever. Her leukemia remains in remission 30 months from diagnosis and she has no evidence of residual aspergillosis. She is attending high school. Her bilirubin levels are about 20–25 μmol/L, with transaminases, urea, and creatinine levels within normal limits. Total cumulative dose of ABCD was 2.3 g/kg and voriconazole 0.9 g/kg. The course of the disease, including lab parameters and treatment given, is summarized in Figure 2.

DISCUSSION

Brain abscess due to invasive *Aspergillus* spp. is a rare and devastating infection and successful treatment of pediatric patients has been infrequently described in literature [10]. Major risk factor for invasive aspergillosis is persistent and profound neutropenia, typically encountered in children receiving intensive cytotoxic chemotherapy for leukemias, lymphomas, and bone marrow transplantation. Disseminated aspergillosis in granulocytopenic and corticosteroid-treated patients is usually an ominous complication of pulmonary infection. The CNS is the most common target organ of hematogenous disseminated aspergillosis. Manifestations of CNS aspergillosis include focal seizures, hemiparesis, and cranial nerve palsies [11]. However, evidence of coexisting lung infection was documented only in 30–48% cases of aspergillus cerebral abscess [4]. In our patient there was no radiological or microbiological evidence (from tracheal secretion) supporting the diagnosis of pulmonary aspergillosis. Open lung biopsy, or bronchoalveolar lavage would have probably solved the question, but in the absence of radiological pulmonary lesion it would not change the treatment, so those investigations were not performed.

The prognosis of aspergillus cerebral abscess is generally poor, regardless of therapy [3, 4]. In selected cases success was attributed to prolonged antifungal therapy using adequate drug dosing [3, 12]. Recent reports suggest improved survival with voriconazole [5].

Voriconazole is a new triazole with broad-spectrum activity against various yeasts and molds, including aspergillus species. Its ability to treat invasive aspergillosis and reduce associated mortality has been demonstrated in large randomized trials [13–15].

However, the need to continue antileukemic therapy with vinca alkaloids, especially in the setting of a child with liver failure, made the choice more complicated, as voriconazole inhibits the cytochrome P450 isoenzymes CY3A4, CYP2C9, and CYP2C19, hence a high risk of potentially serious drug interactions [16].

Another issue is that pediatric dosing of voriconazole still being worked out [7]. However, optimal dosing of amphotericin B lipid formulations (mainly amphotericin B lipid complex and amphotericin B colloid dispersion) remains a matter of ongoing studies as well [9, 17, 18]. As liposomal amphotericin B (AmBisome) is not licensed in The Czech Republic, and taking into consideration preexisting liver impairment, expected duration of the treatment, and size of the particle, ABCD was chosen as agent of choice.

Regarding etiology of the acute liver failure, we can only speculate, as even the open biopsy failed to bring the diagnostic clue. Asymptomatic hereditary lesions (e.g., Gilbert syndrome) might be considered, together with leukemic liver infiltration and superposition of hepatotoxic chemotherapy.

As the galactomannan detection and DNA sequence detection became available for her only during the ongoing treatment, results are not discussed here.

Recent studies firmly establish erythropoietin (EPO) as a multifunctional molecule, with neuroprotective properties [19]. The extent of EPO contribution to excellent neurological and neurocognitive recovery of our patient remains speculative.

We believe that clinical, radiological, and histological picture in our patient was most consistent with presumptive *Aspergillus* spp. infection. Other fungal organisms may also invade the body, but *Aspergillus* spp. are the only nonyeast, nonzygomycete that grow in deep tissue. Its characteristic brush-like appearance, together with nonsegmental elongated conidiophores, or stock, as well as the clinical response to amphotericin B distinguishes it from other species such as *Penicillium*. Although we were unable to culture *Aspergillus* spp. (which is common phenomenon in *Aspergillus* infection), the histological findings in our patient are very unlikely to represent any other fungal species.

What we can learn the following from this case:

1. Life-threatening fungal infection may occur early during induction treatment. MR-guided biopsy of suspected brain lesion may provide accurate tissue diagnosis and is feasible even in sick leukemia patients during remission induction and allows for intralesional local instillation of drugs needed.
2. Prolonged combined or sequential antifungal therapy is feasible in the setting of ongoing curative antileukemic treatment complicated with acute, fulminant, liver failure.
3. Renal toxicity of long-term amphotericin B lipid formulations together with vigorous hydration is clinically acceptable and allows for prolonged, effective antifungal therapy, even together with nephrotoxic chemotherapy and antibiotics.

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Successful Treatment of Life-threatening Candida Peritonitis in a Child With Abdominal Non-Hodgkin Lymphoma Using Efungumab and Amphotericin B Colloid Dispersion

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Summary: Invasive fungal infections are serious complications of cancer therapy. We present a case report of a 12-year-old boy diagnosed with abdominal non-Hodgkin lymphoma and fecal and Candida peritonitis during induction chemotherapy. The invasive mycosis was managed using a combined approach of systemic antifungal agents including efungumab and surgical interventions. Efungumab, a recombinant antibody that inhibits extracellular heat shock protein 90, was used in combination with amphotericin B colloid dispersion after the failure of standard approaches.

Key Words: Candida peritonitis, child cancer, efungumab

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Invasive fungal infections (IFIs) are significant cause of morbidity and mortality in immunocompromised patients. The incidence of IFIs in the population of hematology and oncology patients is increasing, even in non-transplant setting. For example, an 11-year review of over 1000 patients treated at a single hospital in the US showed a linear increase in IFI incidence from 2.9% in 1996 to 7.8% in 2001.[1] *Candida* and *Aspergillus* strains continue to be the main sources of infection, but an increase in IFIs caused by zygomycetes has been observed.[2] The most important risk factors for invasive candidiasis (IC) include hematologic malignancy, treatment with steroids and glycopeptides, prolonged neutropenia, graft-versus host disease, and hyperglycemia. Early and reliable diagnosis remains a problem for all IFIs.

Despite the use of aggressive systemic antifungal treatment, the mortality rate in patients with IC is significant. A systematic review of case-control studies reported mortality rates attributable to candidaemia ranging from 5% in a US intensive care unit to 71% in liver-transplant recipients.[3] Liposomal amphotericin B and caspofungin remain the gold standard for empirical therapy of neutropenic patients,[4–7] but azole antifungals (fluconazole and itraconazole) or other echocandines are frequently used in confirmed cases of patients with candidiasis where in-vitro sensitivity was detected.[8] However, intensive antifungal therapy may interfere significantly with anti-tumor treatment, which may lead to treatment interruption and an increased risk of cancer recurrence. Efungumab is a recombinant human antibody against heat-shock protein 90 (HSP90).[9] It has demonstrated significant efficacy against IC in combination with liposomal amphotericin B therapy.[10]

In the following case report, we present our experience with life threatening Candida peritonitis in a child with abdominal non-Hodgkin lymphoma achieving clinical control only after initiating efungumab.

CASE REPORT

At the April 2006, after several weeks of digestive problems, a 12-year-old male patient with mild anemia (hemoglobin, 9.3 g/dL), white blood cell count of $12 \times 10^3/\mu L$, and platelet count of $453 \times 10^3/\mu L$, was diagnosed with a bulky abdominal tumor infiltrating intestinal loops and the mesentery (Fig. 1).

Abdominal surgery showed unresectable tumor and mesenterial lymph node biopsy revealed non-Hodgkin diffuse large B-cell lymphoma. Iliac crest bone marrow biopsy showed no lymphoma cells and cerebrospinal fluid was free of blasts as well. As radiologic studies (including positron emission tomography) did not find any other lymphoma location, the mass in abdomen was the only diseased site (stage III).

Treatment was started with oral dexamethasone 10 mg/m^2/d for 10 days, low-dose cyclophosphamide 200 mg/m^2/d for 2 days, and a single shot of intrathecal cytarabine 30 mg + methotrexate 12 mg in accordance with the pre-phase of the Non-Hodgkin Lymphoma Berlin-Frankfurt-Munchen 95 Protocol[11] (May 2, 2006

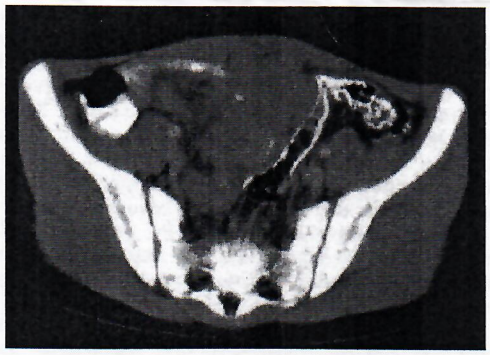

FIGURE 1. Tumor growing among intestinal loops (computed tomography scan).

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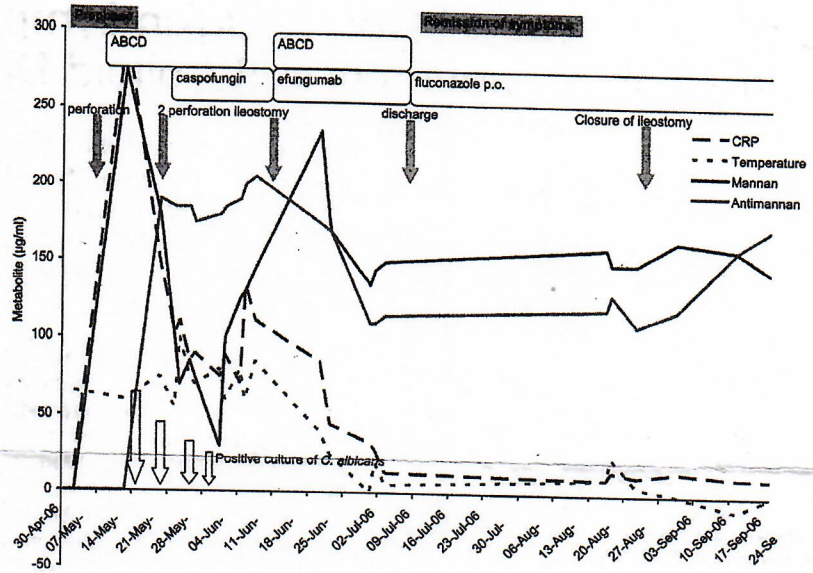
TABLE 1. Time Graph (Weekly) of Clinical and Laboratory Events



ABCD indicates amphotericin B colloid dispersion; *C. albicans*, *Candida albicans*; CRP, C-reactive protein; PO, orally.

to May 12, 2009). The patient's condition began to deteriorate and we could not proceed with further chemotherapy. Radiography ruled out lymphoma progression and suggested multiple perforations of the intestine. Emergency surgical revision of the abdominal cavity led to suture of the 2 perforations on the terminal ileum and the installation of drain due to stercoral peritonitis.

Antibiotics (meropenem, vancomycin, and metronidazole at standard recommended doses) were given in combination. After a short period of improvement, the patient's condition deteriorated again. Cultures from the abdominal drain were positive for *Candida albicans*, and serum levels of mannan and antimannan were elevated (Table 1). Despite systemic anifungals including amphotericin B colloid dispersion (ABCD) empirically, followed by caspofungin + fluconazole after cultures came positive for *Candida* species, the patient's overall condition continued to deteriorate. Surgical revision of the distal ileum found other spontaneous multiple perforations there, this time with numerous intra-abdominal abscesses. The resected terminal ileum showed diffuse fungal peritoneal abscesses with the evidence of nonbranching pseudohyphae and *Candida* blastocysts in each of biopsy sites and no lymphoma infiltrates present.

The abscess cavity in the pelvis was also washed out with fluconazole solution (100 mg twice a day). Karnofsky performance status of our patient was not more than 20% to 30% with continuing deterioration. We then ceased caspofungin treatment and administered efungumab 1 mg/kg twice daily for 10 days in combination with ABCD, as the standard antimycotic therapy was felt to be a failure. The fever gradually subsided and after 5 days of efungumab and ABCD combination therapy, culture samples of *Candida* were negative and the levels of mannan and antimannan in the serum had declined (Table 1). Progressive clinical improvement could be seen from day 2 of efungumab treatment, and gastrointestinal function improved markedly—body weight decline ceased.

The patient was discharged from hospital 4 weeks after the efungumab commencement with oral fluconazole maintenance therapy. Follow-up cultures confirmed complete clearance of *Candida*. Complete remission of the abdominal non-Hodgkin lymphoma was achieved within the BFM 95 pre-phase treatment only and despite the 27 weeks of gap, when no chemotherapy was given. On October 2, 2006, we started with single course of R-COP (according to Children's Oncology Group protocol ANHL0221), consisting of rituximab 375 mg/m^2 intravenously (IV) on days 1 to 3, cyclophosphamide 600 mg/m^2 IV on day 1, and prednisone 1 mg/kg orally on days 1 to 5. We continued with reduced and modified protocol NHL-BFM 04, R2 (2 × A4 and 2 × B4). Modified course A4: dexamethasone 10 mg/m^2 on days 1 to 5, vincristine 1.5 mg/m^2 on day 1, methotrexate 2 g/m^2 during 24 hours infusion (originally 1 g/m^2 during 4 h infusion), ifosfamide 400 mg/m^2 on days 1 to 5 (50% reduction), cytarabin (ARA-C) 150 mg/m^2 IV on day 4 and 5 not given, and etoposide (VP-16) 100 mg/m^2 IV days 4 and 5 not given as well. Inthrathecal installation of 12 mg of methotrexate + cytarabin 30 mg + prednisolone 10 mg on day 2.

Modified course B4: doses for dexamethasone, vincristine, methotrexate, and intrathecal chemotherapy in doses copying modified course A4, cyclophosphamide 200 mg/m^2 IV on days 1 to 5 and doxorubicine 25 mg/m^2 IV on days 4 to 5. A year later (October 2007), we did not find any evidence of tumor recurrence or reactivation of mycosis and maintenance antimycotic therapy (oral fluconazole) was discontinued. At the last follow-up (event free survival 40 mo), the patient's Karnofsky performance status was 100% and there was no evidence of either lymphoma or mycosis.

DISCUSSION

Our patient presented with a combination of IFI risk factors: hematologic malignancy, chemotherapy, steroid treatment, and previous exposure to broad-spectrum of antibiotics. The localization of lymphoma infiltrates contributed to the malfunction of intestine and fungal colonization. Subsequent perforations of affected terminal ileum with diffuse stercoral and candidal peritonitis colored the clinical picture into severe, life-threatening situation, which was not successfully managed with standard

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antimycotic therapy and repeated surgical interventions. Addition of efungumab to ABCD induced an apparent and sustained improvement of the patient's clinical condition and did not worsen the toxicity profile of ABCD.

Efungumab is a recombinant polyhistidine-tagged human monoclonal antibody fragment with specificity for yeast HSP90 (NKILKVIRKNIVKK epitope of HSP90), expressed by transformed *Escherichia coli* bacteria. Efungumab was derived from HSP90 antibody cDNA from patients recently recovered from IC and was developed for treatment of systemic candidiasis. It consists of the antigen-binding variable domains of antibody heavy and light-chains linked and do not have an Fc component. In a double-blind, randomized study, efungumab in combination with liposomal amphotericin B therapy demonstrated significantly greater efficacy against IC than liposomal amphotericin B monotherapy, and was well tolerated.[10] Our experience of using efungumab in a child with NHL and severe invasive candidiasis is very favorable. This case suggests that efungumab may be successfully used when the course of treatment is complicated and the response to standard therapy is unsatisfactory.

A possible additional antitumor effect of efungumab observed has been reported in a group of female breast cancer patients treated with efungumab for mycosis (NeuTec Pharma Ltd, data on file, clinical trial NCT00217815) (http://www.clinicaltrial.gov/ct2/show/results/NCT00217815). In our particular experience, remission of the lymphoma sustained even if chemotherapy course was disrupted for critical 27 weeks after very short and mild, nonaggressive pre-phase. Beside expected infectious activation of the immune system, we speculate that a possible antitumoral effect of efungumab could have played the role. Patient is in remission for 40 months.

Fungal (*Candida*) peritonitis is relatively rare; however, it should be considered as a differential diagnosis in patients presenting with risk factors for IFI, especially when they are colonized by *Candida*. HSP90 either alone or in combination with other antifungals may be an attractive adjunct to study for antifungal therapy in children with cancer.

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Vinorelbine and continuous low-dose cyclophosphamide as maintenance chemotherapy in patients with high-risk rhabdomyosarcoma (RMS 2005): a multicentre, open-label, randomised, phase 3 trial

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Summary

Background For more than three decades, standard treatment for rhabdomyosarcoma in Europe has included 6 months of chemotherapy. The European paediatric Soft tissue sarcoma Study Group (EpSSG) aimed to investigate whether prolonging treatment with maintenance chemotherapy would improve survival in patients with high-risk rhabdomyosarcoma.

Methods RMS 2005 was a multicentre, open-label, randomised, controlled, phase 3 trial done at 102 hospitals in 14 countries. We included patients aged 6 months to 21 years with rhabdomyosarcoma who were considered to be at high risk of relapse: those with non-metastatic incompletely resected embryonal rhabdomyosarcoma occurring at unfavourable sites with unfavourable age (≥10 years) or tumour size (>5 cm), or both; those with any non-metastatic rhabdomyosarcoma with nodal involvement; and those with non-metastatic alveolar rhabdomyosarcoma but without nodal involvement. Patients in remission after standard treatment (nine cycles of ifosfamide, vincristine, dactinomycin with or without doxorubicin, and surgery or radiotherapy, or both) were randomly assigned (1:1) to stop treatment or continue maintenance chemotherapy (six cycles of intravenous vinorelbine 25 mg/m² on days 1, 8, and 15, and daily oral cyclophosphamide 25 mg/m², on days 1–28). Randomisation was done by use of a web-based system and was stratified (block size of four) by enrolling country and risk subgroup. Neither investigators nor patients were masked to treatment allocation. The primary outcome was disease-free survival in the intention-to-treat population. Secondary outcomes were overall survival and toxicity. This trial is registered with EudraCT, number 2005-000217-35, and ClinicalTrials.gov, number NCT00339118, and follow-up is ongoing.

Findings Between April 20, 2006, and Dec 21, 2016, 371 patients were enrolled and randomly assigned to the two groups: 186 to stop treatment and 185 to receive maintenance chemotherapy. Median follow-up was 60·3 months (IQR 32·4–89·4). In the intention-to-treat population, 5-year disease-free survival was 77·6% (95% CI 70·6–83·2) with maintenance chemotherapy versus 69·8% (62·2–76·2) without maintenance chemotherapy (hazard ratio [HR] 0·68 [95% CI 0·45–1·02]; p=0·061), and 5-year overall survival was 86·5% (95% CI 80·2–90·9) with maintenance chemotherapy versus 73·7% (65·8–80·1) without (HR 0·52 [95% CI 0·32–0·86]; p=0·0097). Toxicity was manageable in patients who received maintenance chemotherapy: 136 (75%) of 181 patients had grade 3–4 leucopenia, 148 (82%) had grade 3–4 neutropenia, 19 (10%) had anaemia, two (1%) had thrombocytopenia, and 56 (31%) had an infection. One (1%) patient had a grade 4 non-haematological toxicity (neurotoxicity). Two treatment-related serious adverse events occurred: one case of inappropriate antidiuretic hormone secretion and one of a severe steppage gait with limb pain, both of which resolved.

Interpretation Adding maintenance chemotherapy seems to improve survival for patients with high-risk rhabdomyosarcoma. This approach will be the new standard of care for patients with high-risk rhabdomyosarcoma in future EpSSG trials.

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Introduction

Rhabdomyosarcoma is the most common soft tissue sarcoma in children and young adults. This form of cancer is nonetheless rare, with an annual incidence of four cases per million in individuals aged 0–19 years and approximately 400 new cases each year in Europe.[1] Although rhabdomyosarcoma is regarded as a tumour typical of paediatric age (with highest incidence before

Research in context

Evidence before this study

We searched PubMed for all randomised trials published in English between Jan 1, 1980, and Dec 1, 2018, involving patients with rhabdomyosarcoma. We also searched for published papers with the search terms "rhabdomyosarcoma" and "maintenance". We did not find any randomised trials investigating the role of maintenance chemotherapy or the duration of chemotherapy in rhabdomyosarcoma.
One non-randomised trial suggested that oral maintenance chemotherapy is better than intravenous high-dose chemotherapy in patients with metastatic rhabdomyosarcoma.

Added value of this study

To our knowledge, this study is the first randomised trial to show some improvement in survival with maintenance chemotherapy (six cycles of intravenous vinorelbine 25 mg/m² on days 1, 8, and 15, and daily oral cyclophosphamide 25 mg/m² on days 1–28) for patients with rhabdomyosarcoma. Maintenance chemotherapy administered to patients with high-risk rhabdomyosarcoma in complete remission after standard chemotherapy improved overall survival and was well tolerated. However, the improvement in disease-free survival was not significant.

Implications of all the available evidence

Maintenance chemotherapy improves survival for patients with high-risk rhabdomyosarcoma and will be further investigated in future European paediatric Soft tissue sarcoma Study Group (EpSSG) trials as the new standard of care for this subgroup.

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age 6 years), around 40% of all cases occur in adults.[2] This aggressive tumour is thought to derive from primitive mesenchymal cells committed to developing into striated muscles, but an origin from endothelial progenitors has also been suggested.[3]

Two main histotypes exist: the embryonal subtype, which accounts for approximately 80% of all paediatric rhabdomyosarcomas, and the more aggressive alveolar subtype, which comprises 15–20% of cases and is characterised by a chromosomal translocation involving the fusion of the transcription factor genes *FOXO1* and either *PAX3* or *PAX7*.

Survival of patients with non-metastatic rhabdomyosarcoma is around 70% with the risk-adapted multimodal treatment strategy. This strategy has been refined since the 1970s as a result of several studies coordinated by international cooperative groups, the largest being the Children's Oncology Group (COG) in the USA and the more recently founded European paediatric Soft tissue sarcoma Study Group (EpSSG).[4] These groups have adopted an alkylating agent (ie, cyclophosphamide or ifosfamide) combined with vincristine and dactinomycin, administered every 3 weeks for 6–10 months,[5,6] as the standard chemotherapy regimen for patients with non-metastatic rhabdomyosarcoma. In a series of randomised trials done in the past five decades, attempts to intensify this chemotherapy regimen have not been successful in improving outcomes.[5–13] These trials have shown that most patients with rhabdomyosarcoma achieve complete remission by the end of their treatment, which also includes surgery, radiotherapy, or both. However, the fact that up to one in three patients relapses within 5–9 months after the end of treatment[5,6] suggests that minimal residual active disease is escaping detection through existing radiological methods and is resistant to standard treatment, and thus remains an obstacle to improving survival outcomes. This obstacle might be overcome by introducing new, more effective drugs or adopting new strategies, or through a combination of these approaches.

When the RMS 2005 trial was planned, evidence was available to suggest that vinorelbine is an effective drug against relapsing rhabdomyosarcoma.[14] Some initial claims had also been made that maintenance chemotherapy might be effective against rhabdomyosarcoma.[15] After a pilot study confirmed the effectiveness of vinorelbine combined with low-dose continuous cyclophosphamide,[16] the EpSSG included this novel regimen in the RMS 2005 study and aimed to investigate whether prolonging treatment with a less intensive but continuous chemotherapy regimen could improve outcomes in patients with high-risk rhabdomyosarcoma.

Methods

Study design and participants

RMS 2005 was an investigator-initiated, prospective, international, phase 3, randomised, controlled, open-label trial done at 102 hospitals in 14 countries (Argentina, Belgium, Brazil, Czech Republic, France, Ireland, Israel, Italy, Norway, Switzerland, Slovenia, Spain, the Netherlands, and the UK; appendix p 1).

See Online for appendix

After undergoing diagnostic work-up, each patient was assigned to a specific risk group based on six prognostic factors according to the EpSSG stratification system (appendix p 7). The high-risk group comprised patients with non-metastatic, incompletely resected, embryonal rhabdomyosarcoma occurring at unfavourable sites, age 10 years or older or with a tumour size larger than 5 cm, or both; those with any non-metastatic embryonal rhabdomyosarcoma with nodal involvement; or those with any non-metastatic alveolar rhabdomyosarcoma without nodal involvement. Patients in the low-risk, standard-risk, and very-high-risk groups were not eligible for this study and were treated according to specific recommendations included in the RMS 2005 study.

Patients in the high-risk group were eligible for enrolment into two consecutive independent randomised trials to investigate the benefit of early dose intensification with doxorubicin and the value of maintenance chemotherapy for patients in complete remission after standard therapy. The results of the first trial have been reported elsewhere.[17] Patients were considered for the second trial independently of whether or not they were included in the first trial. The first trial was closed on Dec 17, 2013. After this date, patients were eligible for enrolment into the second trial only.

Eligibility criteria were age older than 6 months at the time of randomisation to younger than 21 years at the time of diagnosis, a pathologically confirmed diagnosis of rhabdomyosarcoma, no evidence of metastatic lesions at the time of diagnosis, no previous illness preventing treatment, no previous malignancies, and no severe vincristine-related neuropathy. Patients also had to be in complete remission or with minimal abnormalities on imaging studies at the end of the standard treatment. These minimal radiological abnormalities were defined as residual signs compatible with fibrosis (which would not have prompted the clinician responsible for the patient to defer stopping treatment). No central radiological review was in place. Patients had to be randomly assigned within 8 weeks after the end of standard treatment, which was defined as the last day of the ninth chemotherapy cycle, the date of surgery, or the date of the end of radiotherapy if done after the ninth cycle of chemotherapy.

Histopathological material had to be available for central diagnostic review, although risk grouping and randomisation were based on local assessments. Molecular confirmation of the presence of a *PAX–FOXO1* translocation was recommended but not mandatory for alveolar subtyping, and was not always done. Patients were removed from the study only if they withdrew consent or did not comply with study procedures.

The trial was designed and overseen by a trial management committee. An independent data monitoring committee reviewed safety and efficacy during the trial. The study was done in accordance with the Declaration of Helsinki and good clinical practice guidelines. All participating centres were required to obtain written approval from their local authorities and ethical committees, as well as written informed consent from patients or their parents or legal guardians.

Randomisation and masking

Eligible patients were randomly assigned (1:1) to stop treatment or continue with maintenance chemotherapy. Randomisation was done with a web-based system provided by CINECA (Bologna, Italy), a non-profit, interuniversity consortium. Patients were stratified in a block size of four by enrolling country and high-risk subgroup (E, F, and G, as described in the EpSSG risk classification, appendix p 7). Neither investigators nor patients were masked to treatment allocation.

Procedures

The diagnostic work-up comprised CT or MRI scans, or both, of the primary tumour, chest CT scan, radionuclide bone scan, bone marrow aspirates, and biopsy. ^{18}F-fluorodeoxyglucose PET was optional. Primary tumour resection was recommended only if a complete resection was considered feasible without harming the patient; otherwise, a biopsy was obtained to establish the diagnosis.

Patients received nine cycles of the IVA chemotherapy regimen: ifosfamide 3 g/m^2 given as a 3 h intravenous infusion with mesna (3 g/m^2) and hydration on days 1 and 2; vincristine 1·5 mg/m^2 given as a single intravenous injection, weekly during the first 7 weeks then only on day 1 of each cycle (maximum dose 2 mg); and dactinomycin 1·5 mg/m^2 on day 1 given as a single intravenous injection (maximum dose 2 mg). From Oct 1, 2005, to Dec 17, 2013, patients were invited to participate in the randomised trial comparing standard IVA with IVADo (IVA plus doxorubicin 30 mg/m^2 on days 1 and 2 in the initial four cycles of chemotherapy).[17] After the trial closed on Dec 17, 2013, the trial management committee recommended treating patients with high-risk rhabdomyosarcoma with nine cycles of IVA (ie, the standard treatment). Local treatment of the primary tumour—including surgery, radiotherapy, or both—was planned after assessing tumour response at week 9, and was implemented at week 13. When a residual mass was identified, surgical resection was encouraged if free margins were achievable without organ or functional impairment. Marginal resection at sites where complete resection was deemed unfeasible was acceptable, provided it was always followed by radiotherapy.

Radiotherapy was the only possible local treatment for patients not able to undergo to secondary surgery because of the tumour's location (eg, parameningeal rhabdomyosarcoma). Radiotherapy doses varied from 41·4 Gy to 50·4 Gy, depending on tumour histology, response to chemotherapy, and surgical outcome. A boost of 5·4 Gy to the residual tumour was recommended for large tumours responding poorly to chemotherapy.

After the ninth cycle of chemotherapy, a full assessment of the tumour was done and patients meeting eligibility criteria were invited to participate in the maintenance chemotherapy trial. Patients were randomly assigned (1:1) to either stop treatment or continue with six 4-week cycles of intravenous vinorelbine 25 mg/m^2 on days 1, 8, and 15 and oral cyclophosphamide 25 mg/m^2 per day given continuously for 24 weeks. This treatment was given on an outpatient basis. In the event of neutropenia ($<1\times10^9$ neutrophils per L) or thrombocytopenia ($<80\times10^9$ platelets per L), or both, during the maintenance therapy phase, cyclophosphamide was stopped until the cell counts recovered, and the third dose of vinorelbine in the subsequent course also withheld if necessary.

If further haematological toxicity occurred, the dose of vinorelbine was reduced to 66% of the full dose on

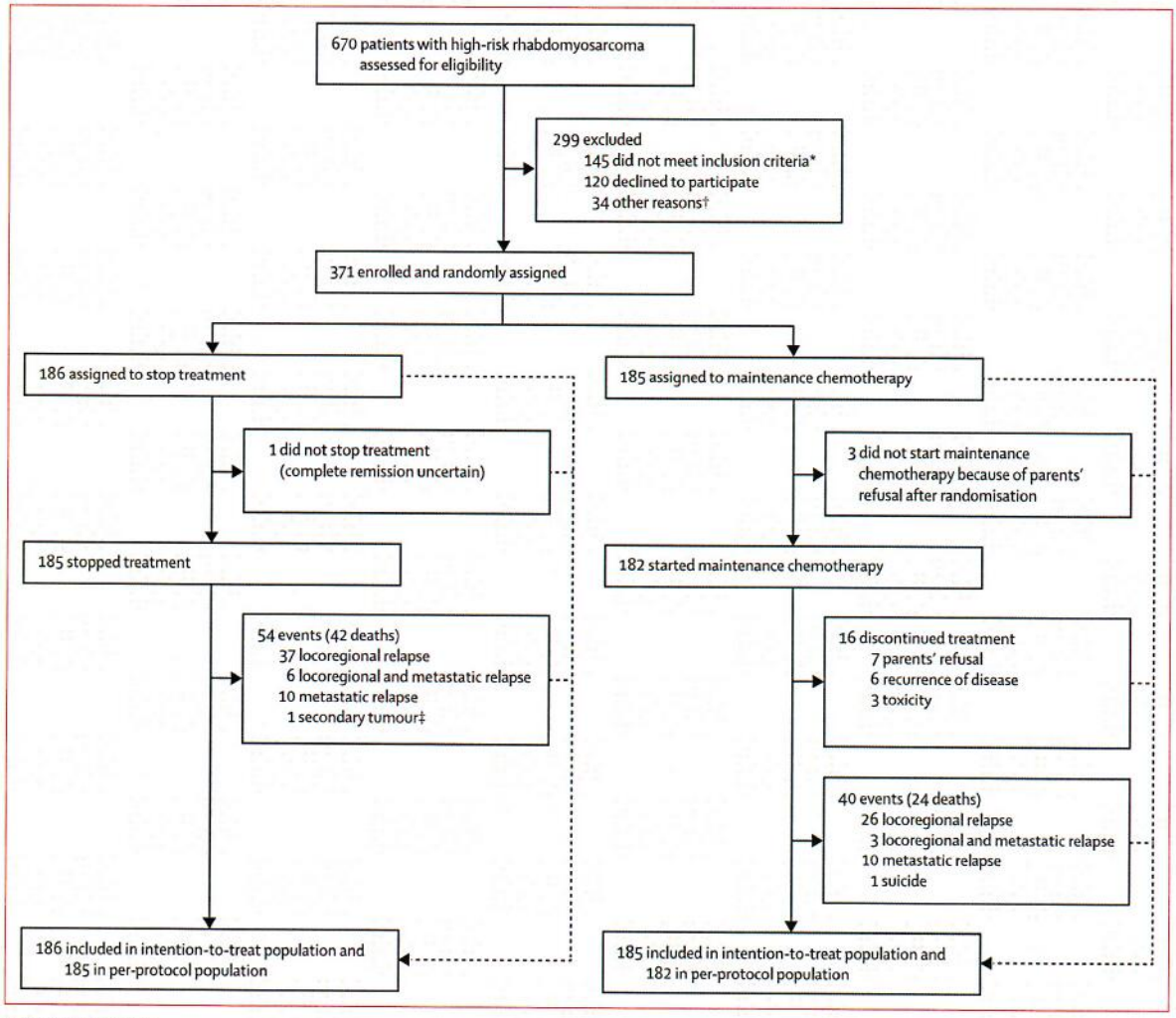

Figure 1: **Trial profile**

*Nine patients were aged older than 21 years at diagnosis, 81 were not in complete remission at the end of standard treatment, 18 had vincristine neuropathy, and in 37 the interval between the end of treatment and the evaluation for the second randomisation was longer than 8 weeks. †27 exclusions were due to the physician's decision, one due to the patient's condition, and six due to organisational reasons. ‡High-grade glioma.

days 1 and 8 (and the third dose omitted), to minimise interruptions in treatment.

Adverse events were monitored at least weekly, and were assessed according to National Cancer Institute Common Toxicity Criteria, version 3. All patients were monitored for possible tumour relapse with CT or MRI scans every 3 months during the first year, every 4 months during the second and third year, and yearly in the fourth and fifth year.

Outcomes

The primary outcome was disease-free survival, which was assessed by the investigator at each centre and not centrally reviewed, and was defined as the time from randomisation to tumour relapse or death from any cause or time of the latest follow-up in patients without an event. Secondary outcomes were overall survival, measured as the time from randomisation to death from any cause, or time to the latest follow-up in patients without an event, and toxicity. Median follow-up time is reported for patients who were alive at the time of data cutoff.

Statistical analysis

The trial was originally designed to enrol 388 patients and observe 200 events to detect an absolute increase in 3-year disease-free survival from 55% in patients who stopped treatment to 67% in those receiving maintenance chemotherapy. This difference would correspond to a relative reduction in the proportion of relapse of 33% in the maintenance treatment group, with 80% statistical power and an alpha of 5% (two-sided log-rank test). The sample size was calculated for a three-step, group sequential design (two interim analyses plus the final analysis) with an O'Brien-Fleming efficacy boundary and the Harrington-Fleming-O'Brien process

	Stop treatment group (n=186)	Maintenance chemotherapy group (n=185)
Age at diagnosis, years		
≤1 year	2 (1%)	11 (6%)
>1–9 years	143 (77%)	136 (74%)
10–17 years	36 (19%)	34 (18%)
≥18 years	5 (3%)	4 (2%)
Sex		
Female	82 (44%)	80 (43%)
Male	104 (56%)	105 (57%)
Histology of rhabdomyosarcoma		
Alveolar	62 (33%)	61 (33%)
Botryoid	5 (3%)	11 (6%)
Embryonal	113 (61%)	109 (59%)
Not otherwise specified	4 (2%)	2 (1%)
Spindle cells or leiomiomatous	2 (1%)	2 (1%)
Pathology		
Favourable	120 (65%)	122 (66%)
Unfavourable	66 (35%)	63 (34%)
Presence of *FOXO* and *PAX3* or *PAX7* translocation		
No	85 (46%)	102 (55%)
Yes	41 (22%)	43 (23%)
Investigation not done	60 (32%)	40 (22%)
Post-surgical tumour staging (IRS)		
Group I*	5 (3%)	5 (3%)
Group II	20 (11%)	21 (11%)
Group III	161 (86%)	159 (86%)
Primary tumour invasiveness		
T1: localised to the organ or tissue of origin	88 (47%)	72 (39%)
T2: extending beyond the tissue or organ of origin	97 (52%)	108 (58%)
Tx: insufficient information about the primary tumour	1 (1%)	5 (3%)

(Table 1 continues in next column)

(Continued from previous column)

	Stop treatment group (n=186)	Maintenance chemotherapy group (n=185)
Tumour size		
≤5 cm	61 (33%)	52 (28%)
>5 cm	125 (67%)	130 (70%)
Not evaluable	..	3 (2%)
Regional lymph node involvement		
N0: no evidence of lymph node involvement	154 (83%)	148 (80%)
N1: evidence of regional lymph node involvement	29 (16%)	31 (17%)
Nx: no information about lymph node involvement	3 (2%)	6 (3%)
Site of origin of primary tumour		
Orbit	7 (4%)	5 (3%)
Head and neck non-parameningeal	11 (6%)	14 (8%)
Parameningeal	56 (30%)	64 (35%)
Bladder prostate	25 (13%)	27 (15%)
Genitourinary non-bladder prostate	5 (3%)	7 (4%)
Extremities	36 (19%)	27 (15%)
Other sites	46 (25 %)	41 (22%)
Subgroup risk		
E	91 (49%)	91 (49%)
F	29 (16%)	31 (17%)
G	66 (35%)	63 (34%)

Data are n (%). IRS=Intergroup Rhabdomyosarcoma Studies. *All IRS group I patients had alveolar histology.

Table 1: **Clinical characteristics of randomised patients by treatment group**

of repeated testing of the alternative hypothesis at an alpha level of 0·005 for futility monitoring. Since the number of patients enrolled and the number of events were lower than planned, on Dec 1, 2011, the independent data monitoring committee recommended re-estimating the sample size and extending the recruitment period, reducing the hazard ratio to be detected to 0·5, and increasing the statistical power to 87%. Based on these assumptions, a new sample size of 370 patients and 79 events, and an interim analysis after observing 50% of the events was planned. At the time of the planned interim analysis in December, 2012, the independent data monitoring committee recommended continuing randomisation as planned. Accrual of patients ended on Dec 21, 2016, and data collected up to Nov 2, 2017, were analysed. The baseline characteristics of the treatment groups were compared with the χ^2 test. Survival probabilities were estimated according to the intention-to-treat principle (ie, including patients in the group to which they were assigned, whether or not they actually received the allocated treatment), by use of the Kaplan-Meier method and the two-sided stratified log-rank test, adjusting for the stratification factors at randomisation to compare the treatment groups at a significance level of 5%. A sensitivity analysis was done for the primary and secondary outcomes in the per-protocol population (ie, eligible patients who received the allocated treatment). 5-year disease-free survival and overall survival were reported with 95% CIs, calculated with Greenwood's method. Hazard ratios (HRs) were estimated with Cox's regression models, adjusted for stratification factors at randomisation, and 95% CIs were calculated according to Wald's method. The proportional hazards assumption was assessed with the score test based on scaled Schoenfeld residuals and was met (p=0·0793). Cox's regression models for disease-free survival and overall survival were estimated to examine possible interactions between treatment efficacy and clinical subgroups. For post-hoc subgroup analyses, no adjustments were made for multiplicity and so these analyses should be interpreted as only being descriptive. Patients who received at least one dose of study treatment were included in the safety analysis,

and toxicities were analysed according to the actual treatment received. All analyses were done with SAS, version 9.4.

This trial is registered with EUDRACT, number 2005-000217-35, and ClinicalTrials.gov, number NCT00339118.

Role of the funding source

EpSSG designed and coordinated the trial. The funders had no role in the design of the study, data collection, data analysis, data interpretation, or writing of the report. GB, IZ, and GLDS had full access to the raw data and had final responsibility for the decision to submit for publication, on behalf of the EpSSG board members.

Results

Between April 20, 2006, and Dec 21, 2016, 670 patients with characteristics of high-risk rhabdomyosarcoma were assessed for eligibility and 371 eligible patients were randomly assigned: 186 (50%) to stop treatment and 185 (50%) to receive maintenance chemotherapy (figure 1). One patient continued with maintenance chemotherapy despite being randomly assigned to stop treatment because their physician was uncertain as to whether the patient's tumour was in complete remission. Three children randomly assigned to the maintenance treatment group did not start the treatment because of parental refusal afterwards. All four patients were included in the intention-to-treat analysis but were excluded from the per-protocol analysis. Central diagnostic review was done in 282 (76%) patients: 146 (79%) of those who stopped the treatment and 136 (74%) of those who received maintenance chemotherapy. Clinical characteristics of patients were well balanced between the two groups (table 1) and were similar to those of non-randomised patients (appendix p 9). The interval from the end of treatment to randomisation was reasonable and similar in the two groups: median 29 days (IQR 17–42) in the group that stopped treatment and 31 days (22–44) in the group that received maintenance chemotherapy.

The treatment received before randomisation was similar in the two groups: 227 (61%) patients received IVA (120 in the maintenance chemotherapy group and 107 in the stop treatment group), and 144 (39%) received IVADo (65 in the maintenance chemotherapy group and 79 in the stop treatment group). More patients received IVA than IVADo because this was the regimen recommended after the first trial was closed on Dec 17, 2013. Complete data about treatment adherence and toxicity were available for 181 (99%) of the 183 patients who started maintenance chemotherapy (since we did a per-protocol analysis of toxicity, we included one patient who was randomly assigned to stop treatment but received maintenance chemotherapy), which was completed by 165 (90%) of 183 patients. The median time from randomisation to the end of maintenance chemotherapy was 5·75 months (IQR 5·45–5·98). Treatment was interrupted at the request of parents in seven children, because of disease recurrence in six, and because of toxicity in three (neurotoxicity in two [one grade 2 and one grade 3] and bone infection in one [grade 3]). 144 (80%) of 181 patients had at least one cycle

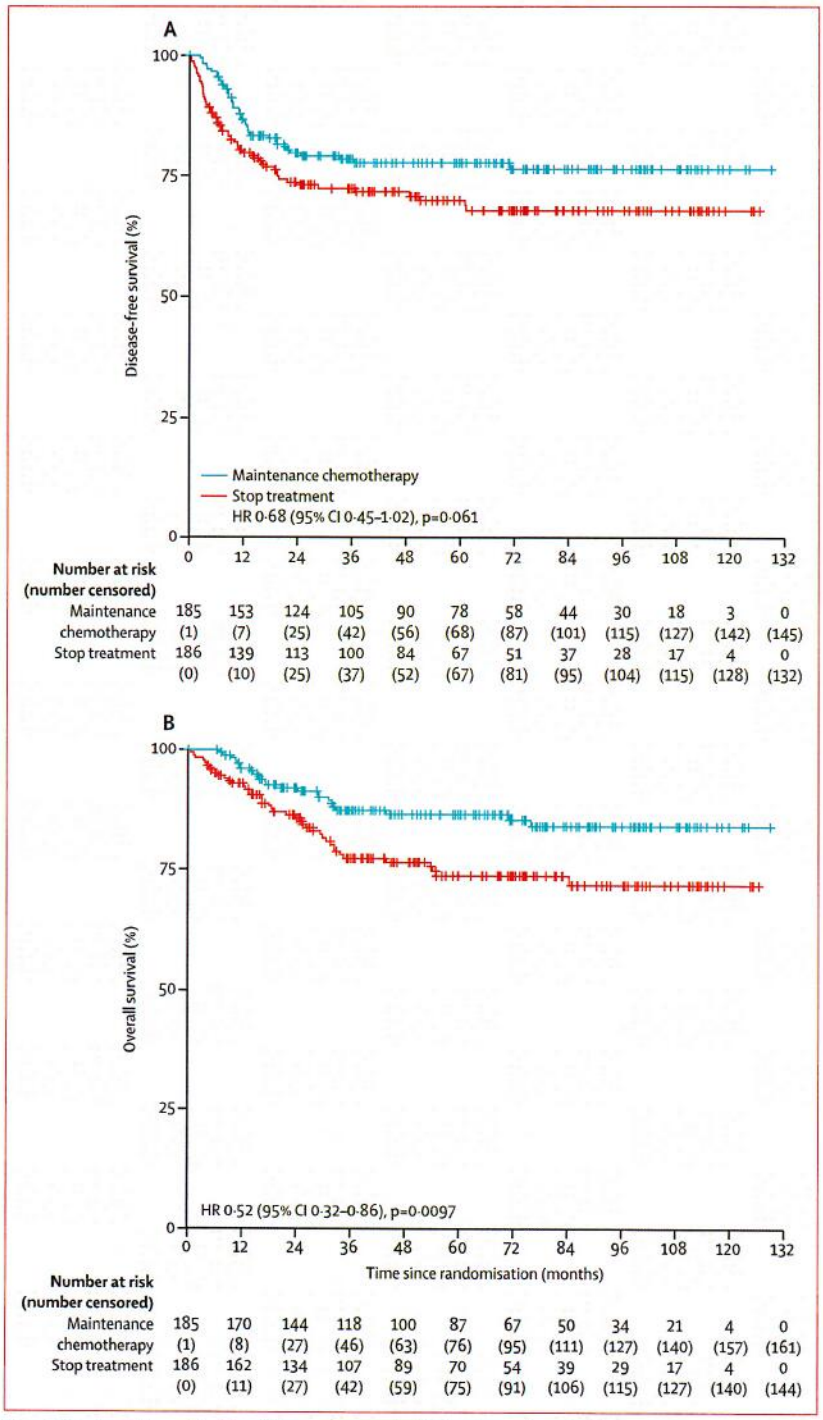

Figure 2: **Kaplan-Meier estimates of disease-free survival (A) and overall survival (B)**
HR=hazard ratio.

	Stop treatment group (n=186)	Maintenance chemotherapy group (n=185)
All events	54	40
Local relapse or regional lymph node relapse	37 (69%)	26 (65%)
Local or regional lymph node relapse and metastasis	6 (11%)	3 (8%)
Metastases	10 (19%)	10 (25%)
Death	1* (2%)	1† (3%)

Data are n or n (%). *Died by suicide. †Died after second tumour (high-grade glioma). One patient who died from a surgical complication and one who died from H1N1 influenza are not reported here because these were not the first events.

Table 2: **First events by randomised group**

	Grade 1–2	Grade 3	Grade 4
Haematological toxicity			
Anaemia	128 (71%)	16 (9%)	3 (2%)
Leucopenia	26 (14%)	86 (48%)	50 (28%)
Neutropenia	16 (9%)	66 (37%)	82 (45%)
Thrombocytopenia	28 (16%)	1 (1%)	1 (1%)
Non-haematological toxicity			
Cardiac	1 (1%)	..	..
Infection	33 (18%)	56 (31%)	..
Fever and neutropenia	4 (2%)	44 (24%)	..
Fever without neutropenia	26 (14%)	9 (5%)	..
Other infection	3 (2%)	3* (2%)	..
Nephrotoxicity	14 (8%)	1 (1%)	..
Neurology	21 (12%)	2 (1%)	1 (1%)†
Nausea or vomiting	34 (19%)	1 (1%)	..
Gastrointestinal	41 (23%)	9 (5%)	..
Allergy	4 (2%)	..	..
Dermatological	7 (4%)	1 (1%)	..
Other‡	37 (20%)	1 (1%)‡	..

Data are n (%). Toxicity data were only available for 181 patients. *Bone infection in one patient and pulmonary infection in two patients. †Steppage gait with limb pain that completely resolved after 1 month. ‡Hypokalemia.

Table 3: **Adverse events reported in 181 patients during maintenance chemotherapy**

modification: drug doses were reduced in accordance with the recommendations of the protocol to deal with neutropenia or thrombocytopenia in 74 (51%) patients; reduced because of toxicity in 63 (44%), and reduced for other reasons in seven (5%; appendix p 11).

At the time of data cutoff, the median follow-up for patients who were still alive was 60·3 months (IQR 32·4–89·4), so the 5-year results are reported here. In the intention-to-treat population, 5-year disease-free survival was 77·6% (95% CI 70·6–83·2) for patients who received maintenance chemotherapy versus 69·8% (62·2–76·2) for patients who stopped treatment (HR 0·68 [95% CI 0·45–1·02]; p=0·061). 5-year overall survival was 86·5% (95% CI 80·2–90·9) for patients who received maintenance chemotherapy versus 73·7% (65·8–80·1) for patients who stopped treatment (HR 0·52 [95% CI 0·32–0·86]; p=0·0097; figure 2). 367 patients met the criteria for the per-protocol analysis. 5-year disease-free survival was 69·6% (95% CI 62·0–76·0) in the group given no further treatment and 77·8% (70·8–83·4) in the group given maintenance chemotherapy (HR 0·67 [95% CI 0·44–1·01]; p=0·053). 5-year overall survival was 73·5% (95% CI 65·6–79·9) in the group given no further treatment and 86·3% (79·9–90·8) in the group given maintenance chemotherapy (HR 0·53 [95% CI 0·32–0·87]; p=0·011).

94 (25%) of 371 patients had a relapse event, with local and metastatic relapses similarly distributed in the two groups (table 2). The median time to relapse calculated from the randomisation date to the event was 6·9 months (IQR 3·0–16·1) in the group given no further treatment and 10·1 months (6·9–15·4) in the maintenance chemotherapy group.

66 (18%) patients died: 42 (23%) of 186 in the group given no further treatment and 24 (13%) of 185 in the maintenance therapy group. All deaths were related to tumour relapse except for two patients in the group given no further treatment (one from a surgical complication after a local relapse and one from suicide), and two in the maintenance chemotherapy group (an infection with H1N1 influenza after metastasis to the lung in one patient and high-grade glioma occurring as a second tumour 69·7 months after rhabdomyosarcoma in the other patient).

A post-hoc exploratory subgroup analysis, taking into account clinical variables known to be of prognostic value—such as age at diagnosis, histological subtype, primary tumour invasiveness, nodal involvement, tumour size and site, and Intergroup Rhabdomyosarcoma Studies group—showed no differences in any subgroup of patients between the two groups (appendix p 12).

The randomised comparison between the IVA and the IVADo regimens, which was part of the RMS 2005 study, did not differ significantly in terms of disease-free survival and overall survival between the two groups.[17] In a post-hoc analysis, a possible interaction between the initial standard chemotherapy (IVA or IVADo) and any subsequent maintenance chemotherapy was ruled out with Cox's regression models, for both disease-free survival (p=0·54) and overall survival (p=0·84; appendix p 13).

In view of the greater difference between the two groups in overall survival than in disease-free survival, a post-hoc analysis was done on the distribution of the characteristics that might have a prognostic effect for patients with a relapse: all variables were found to be well balanced between the two groups (appendix p 14). We noted a difference among countries in the number of patients considered in complete remission at the end of standard treatment and therefore eligible for the randomised study (appendix page 8). This difference was more evident in countries that enrolled a small number of patients.

Toxicity data are summarised in table 3. Grade 4 neutropenia was the most common adverse event, occurring in 82 (45%) patients, and grade 3 infection was reported in 56 (31%). 136 (75%) of 181 patients had grade 3–4 leucopenia, 148 (82%) had grade 3–4 neutropenia, 19 (10%) had anaemia, and two (1%) had thrombocytopenia. One patient (1%) had grade 4 non-haematological toxicity (neurotoxicity). Two treatment-related serious adverse events occurred: one patient had inappropriate antidiuretic hormone secretion and the other had a severe steppage gait with limb pain. Both events were resolved but maintenance treatment was permanently discontinued in the patient who had inappropriate antidiuretic hormone secretion.

Discussion

The results of this international randomised trial show that maintenance chemotherapy with vinorelbine and low-dose oral cyclophosphamide after standard treatment improves overall survival of patients with high-risk, non-metastatic rhabdomyosarcoma. In three decades of international cooperative trials,[4–13] this randomised study is, to the best of our knowledge, the first to show a survival benefit related to an experimental chemotherapy regimen.

The improvement in overall survival was significant and clinically important, whereas the improvement in disease-free survival—the primary endpoint—was not. However, in the per-protocol analysis (in which only a few patients were excluded in comparison with the intention-to-treat analysis) both disease-free and overall survival were significantly improved with maintenance chemotherapy, thus lending support to the activity of this regimen. Whether or not post-relapse treatment had any effect on survival could not be verified, because patients received different types of chemotherapy, with or without radiotherapy or surgery, or both. Previous studies identified factors that predict survival after relapse[18] and these factors were well balanced in our study population. Maintenance chemotherapy might have led to selection of patients in some way (eg, outcomes after late relapses are reported to be better, and in our cohort the median time to an event was 3 months later in patients randomly assigned to maintenance chemotherapy than in those assigned to stop treatment). Finally, the effectiveness of maintenance chemotherapy in the experimental group is also supported by the results of the per-protocol analysis, which show a significant improvement in disease-free survival in patients who received further treatment.

We were unable to identify subgroups of patients in whom maintenance chemotherapy was more effective and we ruled out any possible influence of previous treatments.

A limitation of the study was the high proportion of potentially eligible patients who were not randomly assigned, mainly because of parents' refusal. However, not including these patients is unlikely to have influenced the results substantially because the characteristics of non-randomised patients were similar to those of randomised patients. The inability to achieve complete tumour remission at the end of standard treatment, based on radiology investigations, was another reason for exclusion of several patients from this study. No central radiological review was in place but national coordinators were available to discuss difficult cases. We found some differences among countries in the number of patients not considered in complete remission, but randomisation was stratified by enrolling countries, thus preventing possible bias.

When the EpSSG RMS 2005 protocol was developed, the idea of a possible effect of maintenance therapy was based on sparse clinical evidence. The use of low-dose chemotherapy to maintain remission is a key concept in paediatric acute lymphoblastic leukaemia,[19] but such a strategy has been rarely investigated in solid tumours. In paediatric soft tissue sarcomas, the German Cooperative Group used oral maintenance chemotherapy (trofosfamide plus etoposide or idarubicin) as an alternative to high-dose chemotherapy with stem-cell rescue after standard therapy in children with metastatic disease. Although the study had some major limitations (ie, it was not randomised and the treatment was chosen at the discretion of the physician), it did suggest a promising role for maintenance chemotherapy.[15]

When the EpSSG RMS 2005 trial was developed, the activity of vinorelbine as a single agent in rhabdomyosarcoma had been documented in a single study,[14] which was subsequently supported by a second study showing 36% of patients achieving a response in relapsing rhabdomyosarcoma.[20] Cyclophosphamide had already been used successfully at low doses (2·5 mg/kg per day for up to 2 years).[7,8] A potentially anti-angiogenic and immunomodulatory effect has been suggested for both vinca alkaloids and continuous low-dose cyclophosphamide.[21–25] Additionally, these two drugs were not part of the initial chemotherapy regimen adopted in the RMS 2005 study, making chemoresistance issues less likely. All these reasons made this combination ideal as a maintenance therapy in the RMS 2005 trial. Moreover, before starting the trial, the new combination was tested in a pilot study, which showed that it was well tolerated and active.[16] This result was later confirmed by a larger phase 2 study.[26]

Our trial shows the feasibility of delivering this drug combination after standard chemotherapy. More than 90% of patients completed the treatment, although 80% required drug dose modification according to the protocol guidelines to avoid excessive myelosuppression. Although administration of cyclophosphamide should not increase the risk related to the cumulative doses of ifosfamide previously administered, the risk of long-term toxicity remains to be established, particularly the possibility of an increased risk of gonadal damage and secondary malignancies.

The observed improvement in overall survival could be explained in many ways. Prolonging chemotherapy might have improved survival in children with a small amount of residual disease remaining at the end of standard treatment. The optimal duration of chemotherapy for rhabdomyosarcoma has yet to be established. The duration has gradually decreased over the years, without apparently impairing the results of treatment. For example, treatment duration was reduced from 2 years to 1 year from the IRS-I study to the IRS-IV study,[7–10] and most patients receive 42 weeks of treatment in contemporary COG protocols. In Italian studies, treatment duration was reduced from 52 weeks or 78 weeks (depending on the risk group) in the first study to 22–37 weeks in the second, and 25 weeks in the third, without jeopardising patient outcomes.[27] However, the results of a retrospective analysis on extremity rhabdomyosarcoma, pooling data from US and European protocols, showed an improved outcome for patients treated with longer periods of chemotherapy compared with those who received a shorter duration of treatment.[28] Other differences in treatment strategies used by the various cooperative groups might, however, also account for these results.

An alternative hypothesis to explain the improved outcome for patients treated with maintenance therapy might be the effectiveness of the drugs involved (ie, vinorelbine and low-dose cyclophosphamide). In previous studies, the proportion of patients achieving a response to single-agent vinorelbine was similar to those achieving a response to vinorelbine combined with low-dose cyclophosphamide,[14,16,20,26] so the additive effect of the combination is unclear. But fully assessing the relative contribution of each drug by comparing the results of different studies is difficult. That said, the combined regimen might have killed any residual tumour cells resistant to the drugs administered during the standard treatment. This benefit seemed to be more evident in preventing locoregional rather than metastatic events. Since locoregional relapse is the most frequent cause of treatment failure and death, the effect of maintenance treatment might have been more evident in this group of patients.

When the RMS 2005 trial was started, the possibility of adding the effect of a metronomic approach to the effect of conventional chemotherapy was appealing. The prolonged exposure of tumour cells to chemotherapy, together with possible anti-angiogenic and immunomodulatory effects, are reportedly behind the mechanism of action of drugs given continuously at low doses.[24,25]

Finally, the effectiveness of maintenance chemotherapy could also relate to the compound effect of a longer period of chemotherapy and the efficacy of the drugs used in the maintenance phase.

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In the RMS 2005 trial, the role of maintenance chemotherapy was investigated in patients with high-risk disease (according to the EpSSG definition) with no evidence of an active residual tumour at the end of standard treatment. Although additional maintenance chemotherapy might not be considered necessary in patients with low-risk or standard-risk rhabdomyosarcoma, which has an excellent prognosis with standard treatment, this new strategy might be of benefit for children at higher risk of failure (ie, those with metastatic disease at diagnosis).

Maintenance chemotherapy was designed by taking into account the overall structure of the RMS 2005 trial and we do not know whether or not this strategy could be adopted for patients treated according to other protocols with a longer treatment duration (eg, COG protocols). This strategy might lead to an overall treatment duration that is less acceptable to patients and additional concerns about long-term toxicity. One option is to consider maintenance therapy in lieu of several more intense cycles of chemotherapy, to minimise toxicity while maintaining outcomes.

The role of maintenance therapy in the treatment of rhabdomyosarcoma, and possibly of other paediatric solid tumours, needs to be better elucidated. Further studies have been planned by the EpSSG to investigate the effectiveness of this strategy in patients with metastatic disease, whose prognosis is still largely unsatisfactory. The possible benefit of a longer duration of the maintenance phase will also be addressed in a randomised trial. Different drug combinations could also be investigated, and the mechanism of action behind the effect of maintenance therapies needs to be better understood.

In conclusion, this study showed that maintenance treatment with vinorelbine and low-dose oral cyclophosphamide for patients with high-risk rhabdomyosarcoma in complete remission after standard treatment improves overall survival and is safe and well tolerated. This approach has now been adopted by the EpSSG as the new standard of care for patients with high-risk rhabdomyosarcoma.

Contributors

All authors contributed to the study design, data collection, and interpretation, management of the clinical trial, writing and review of the paper, and approval of the final version. GB acted as principal investigator and was part of the trial management committee, along with CB, MJ, SGM, and AF. GB, GLDS, CB, MJ, AK, HM, SGM, and AF wrote the protocol and organised data collection. JHM, VMC, HG, JC, MC, CD, MBA, PM, and SF coordinated the protocol in the participating countries. GLDS coordinated the data centre and did the statistical analysis with IZ.

Declaration of interests

We declare no competing interests.

Data sharing

Individual participant data are not publicly available since this was requirement not anticipated in the study protocol. The protocol can be requested through the EpSSG website.

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Original Article

Fusion Status in Patients With Lymph Node-Positive (N1) Alveolar Rhabdomyosarcoma Is a Powerful Predictor of Prognosis: Experience of the European Paediatric Soft Tissue Sarcoma Study Group (EpSSG)

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BACKGROUND: Alveolar rhabdomyosarcoma (aRMS) with lymph node involvement (N1 classification) accounts for up to 10% of all cases of RMS. The prognosis is poor, and is comparable to that of distant metastatic disease. In the European Paediatric Soft Tissue Sarcoma Study Group (EpSSG) RMS2005 protocol, patients with a histologic diagnosis of aRMS/N1 received intensified chemotherapy with systematic locoregional treatment. **METHODS:** Patients with aRMS/N1 were enrolled prospectively after primary surgery/biopsy and fusion status was assessed in tumor samples. All patients received 9 cycles of induction chemotherapy and 6 months of maintenance therapy. Local treatment included radiotherapy to the primary site and lymph nodes with or without secondary surgical resection. **RESULTS:** A total of 103 patients were enrolled. The clinical characteristics of the patients were predominantly unfavorable: 90% had macroscopic residual disease after initial surgery/biopsy, 63% had locally invasive tumors, 77% had a tumor measuring >5 cm, and 81% had disease at unfavorable sites. Fusion genes involving forkhead box protein O1 (*FOXO1*) were detected in 56 of 84 patients. Events occurred in 52 patients: 43 developed disease recurrence, 7 had disease that was refractory to treatment, and 2 patients developed second neoplasms. On univariate analysis, unfavorable disease site, tumor invasiveness, Intergroup Rhabdomyosarcoma Study group III, and fusion-positive status correlated with worse prognosis. The 5-year event-free survival rate of patients with fusion-positive tumors was 43% compared with 74% in patients with fusion-negative tumors ($P = .01$). On multivariate analysis, fusion positivity and tumor invasiveness proved to be unfavorable prognostic markers. **CONCLUSIONS:** Fusion status and tumor invasiveness appear to have a strong impact on prognosis in patients with aRMS/N1. Fusion status will be used to stratify these patients in the next EpSSG RMS study, and treatment will be intensified in patients with fusion-positive tumors. ***Cancer* 2018;124:3201-9.**
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KEYWORDS: alveolar rhabdomyosarcoma, lymph node involvement, paired box (*PAX*)-forkhead box protein O1 (*FOXO1*) fusion, prognostic factors, rhabdomyosarcoma.

INTRODUCTION

Rhabdomyosarcoma (RMS) is one of the most frequent extracranial solid tumors diagnosed in children and the most common form of soft-tissue sarcoma diagnosed in children and young adults.[1] The prognosis of patients with localized RMS has improved considerably over time thanks to numerous clinical trials conducted by collaborative groups working in North America (Children's Oncology Group [COG]) and Europe (International Society of Pediatric Oncology [SIOP] Malignant Mesenchymal Tumor Group [MMT], Italian Soft Tissue Sarcoma Committee [STSC], and German Cooperative Soft Tissue Sarcoma Study Group [CWS]). The presence of disseminated disease at the time of diagnosis is

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the most powerful prognostic factor in RMS. Although the probability of cure in pediatric patients with localized disease is >70%, the prognosis of those with distant metastatic disease remains poor.[2-8] In patients with localized disease, clinical and tumor characteristics have been used to classify RMS into different risk categories and to determine treatment intensity. Unfavorable characteristics include alveolar histology, invasive tumor (T2 classification), tumor location, lymph node involvement, tumor size >5 cm, and patient age ≥10 years[9-11] and constitute the basis for the risk stratification system used in the recent European Paediatric Soft Tissue Sarcoma Study Group (EpSSG) RMS2005 study. Previous experience has suggested that patients with alveolar RMS (aRMS) and regional lymph node involvement represent a group with a particularly poor prognosis.[11]

Approximately 70% of patients with aRMS present with the fusion genes paired box 3 (*PAX3*)-forkhead box protein O1 (*FOXO1*) or paired box 7 (*PAX7*)-*FOXO1* as a consequence of the reciprocal chromosomal translocations t(2;13)(q35;q14) or t(1;13)(p36;q14).[12] Recent data have suggested that the *PAX3/7-FOXO1* fusion genes have prognostic significance.[13,14] This observational study reports on the results obtained in this very high-risk population, and focuses on the prognostic role of fusion gene status.

MATERIALS AND METHODS

Patients

The RMS2005 protocol was initiated in October 2005 and opened in 14 countries. Eligibility criteria for inclusion in the RMS2005 protocol were age >6 months to <21 years, a pathologically proven diagnosis of RMS, no evidence of distant metastatic lesions, tumor previously untreated except for primary surgery, no preexisting illness preventing treatment, no previous malignant tumors, and an interval between diagnostic surgery and treatment of ≤8 weeks. Patients with localized aRMS and regional lymph node involvement (N1 classification) were assigned to the very high-risk group according to the EpSSG stratification system. This group is the focus of the current analysis, with particular attention to the group of patients who underwent molecular analysis of *PAX3/7-FOXO1* fusions. Only patients enrolled before December 31, 2013 were included in this analysis to ensure an adequate follow-up. The cutoff date for the analysis was April 4, 2017.

Staging

Disease was staged according to the TNM classification and the Intergroup Rhabdomyosarcoma Study Group (IRS) postsurgical grouping system.[15] Regional lymph node involvement was indicated as N0 or N1 and distant metastases at the time of onset as M0 or M1 based on histologic or clinical/radiologic assessments.

Tumor location was considered favorable if arising from the orbit, genitourinary region other than the bladder or prostate (ie, paratesticular and vagina/uterus), and nonparameningeal head and neck, and was considered unfavorable when arising from any other site.

Regional lymph nodes were defined as those appropriate to the site of the primary tumor. Any evidence of distant lymph node involvement other than these was considered metastasis and patients were treated according to the protocol for those with metastatic disease at the time of diagnosis. Surgical exploration of regional lymph nodes was mandatory in cases of RMS arising in the limbs. In tumors originating in other locations, regional lymph node involvement was determined clinically and by imaging, including magnetic resonance imaging and/or positron emission tomography (PET)-computed tomography scan. In doubtful cases, a lymph node biopsy was recommended. Systematic sentinel lymph node examination was suggested but implemented only at a small number of centers.

Treatment

Patients received intensified initial chemotherapy and additional maintenance chemotherapy with systematic local treatment to the primary and lymph node sites. Induction chemotherapy comprised 4 cycles of 21 days each of ifosfamide at a dose of 3 g/m^2 on days 1 to 2 with mesna; vincristine at a dose of 1.5 mg/m^2 (maximum, 2 mg) on days 1, 8, and 15 in the first 2 cycles and day 1 in cycles 3 and 4; actinomycin D at a dose of 1.5 mg/m^2 (maximum, 2 mg) on day 1; and doxorubicin at a dose of 30 mg/m^2 on days 1 to 2 (IVADo) followed by 5 cycles of 21 days each of ifosfamide at a dose of 3 g/m^2 on days 1 to 2 with mesna, vincristine at a dose of 1.5 mg/m^2 on day 1, and actinomycin D at a dose of 1.5 mg/m^2 on day 1 (IVA) and 6 cycles of 28 days each of maintenance chemotherapy comprising continuous daily oral cyclophosphamide at a dose of 25 mg/m^2 and intravenous vinorelbine at a dose of 25 mg/m^2 on days 1, 8, and 15 of each cycle.[16]

Local treatment after the initial 4 cycles of IVADo (week 13) included delayed (secondary) surgery to remove macroscopic residual tumor and radiotherapy (RT). External beam RT was scheduled to be given to the primary tumor area and the affected lymph node region. Doses varied according to chemotherapy response and surgical results and were administered in 1.8-gray (Gy)

daily fractions. The total dose to the primary tumor in postsurgical IRS group II and group III patients with complete remission after secondary surgery was 41.4 Gy. For patients in IRS group III with incomplete secondary resection or when secondary surgery was not feasible, the total dose was 50.4 Gy with an optional additional boost of 5.4 Gy in 3 fractions for large tumors with poor responses to chemotherapy. RT to the involved lymph nodes was recommended at a dose of 41.4 Gy regardless of surgical resection. Treatment was delivered with megavoltage photons at 1 fraction per day for 5 days per week.

Response was evaluated after initial chemotherapy (week 9) and at the end of treatment by 3-dimensional volumetric assessment using the formula: tumor volume (cm^3) = 0.52 × length (cm) × width (cm) × thickness (cm). Responses were defined as complete response (clinically or histologically confirmed complete disappearance of disease), partial response (at least a two-thirds reduction in tumor volume), minor response (a reduction in tumor volume greater than one-third but less than two-thirds), stable disease (a modification in tumor volume of less than one-third), and progressive disease (an increase in tumor size >30% or the detection of new lesions).

The site of first disease recurrence was defined as local if the tumor recurred at the site of primary disease, lymph node if regional lymph nodes were involved, locoregional in cases of local and lymph node disease recurrence, distant in cases with the appearance of metastatic disease, and combined when locoregional plus metastatic disease recurrence were evident.

Pathology and Biology

Histologic analysis was performed locally at participating EpSSG centers using routine hematoxylin and eosin staining. Following protocol guidelines, a panel of appointed pathologists reviewed 2 to 12 tumor slides from each patient and confirmed the diagnosis of aRMS.

The molecular characterization of aRMS was part of several translational studies to be implemented in the RMS2005 protocol. The analysis was strongly recommended and should be conducted at a single laboratory for each participating national group. However, fusion status data were not available for the entire population because of a shortage of suitable or fresh biologic material. Molecular analysis of the *PAX3/7-FOXO1* fusion was performed by fluorescent in situ hybridization (FISH) in paraffin blocks and/or by reverse transcriptase-polymerase chain reaction (RT-PCR) in frozen tissue. Interphase and metaphase FISH studies for RMS translocations were performed using chromosome 13 cosmids flanking the *FOXO1* gene using a commercial break-apart probe as described.[12] RNA from snap-frozen tumor was assayed by single-round RT-PCR using the primer pairs and conditions as described.[17] Only samples with a sufficient number of tumor cells (>50%) were considered for the analysis. Alternative *PAX3* fusions with partners other than *FOXO1* were not analyzed. Samples with *FOXO1* gene disruption (ie, positive *PAX3-FOXO1, PAX7-FOXO1*, or *FOXO1* with an unknown gene partner) were considered fusion status positive.

Statistical Analysis

Data were collected via a Web-based system and analyzed at Veneto Oncologic Institute (Padova, Italy). Continuous variables were summarized with the median, minimum, and maximum, whereas categorical variables were reported as counts and percentages.

Survival was calculated from the date of diagnosis to the time of the event or last follow-up. Tumor progression, disease recurrence, occurrence of a second malignancy, or death due to any cause were considered for event-free survival (EFS). Overall survival (OS) was measured from the date of diagnosis to the date of death from any cause. Patients who still were alive at the end of the study were censored at the date of the last observation.

Survival probability was computed using the Kaplan-Meier method and heterogeneity in survival among strata of selected variables was assessed with the log-rank test. The 5-year EFS and OS rates of the patient subgroup with available molecular data were reported along with their 95% confidence intervals (95% CIs), computed using the Greenwood formula.

The Cox proportional hazards regression method was used to ascertain whether fusion-positive status may have prognostic significance in this cohort of patients. A stepwise variable selection procedure was applied to the covariates with a P value $\geq .25$ in the univariate analysis. Hazard ratios (HRs) with 95% CIs according to the Wald method were reported for independent selected variables. All data analyses were performed using the SAS statistical package (release 9.4; SAS Institute Inc, Cary, North Carolina).

Ethics

The EpSSG RMS2005 treatment protocol was submitted to the institutional and national review boards of each participating country for review and approval before the enrollment of patients. Written informed consent for participation was obtained from patients, parents, or legal guardians in all cases. The study was conducted in

TABLE 1. Clinical Characteristics of Patients With aRMS and Lymph Node Involvement (N1 Classification)

Characteristic	Molecular Biology Not Performed N=18		Molecular Biology Performed N=85		Total N=103		*P*
	No. of Patients	%	No. of Patients	%	No. of Patients	%	
Age at diagnosis							
<10 y	5	27.8	47	55.2	52	50.5	.0339
≥10 y	13	72.2	38	44.8	51	49.5	
Sex							
Female	6	33.3	35	41.2	41	39.8	.5369
Male	12	66.7	50	58.8	62	60.2	
Postsurgical IRS group							
II	1	5.6	9	10.6	10	9.7	.5124
III	17	94.4	76	89.4	93	90.3	
Tumor invasiveness (T classification)							
T1	5	27.8	33	38.8	38	36.9	.3776
T2	13	72.2	52	61.2	65	63.1	
Tumor size							
a: ≤5 cm	3	16.7	20	23.5	23	22.3	0.7224
b: >5 cm	15	83.3	64	75.3	79	76.7	
x: Not evaluable	-	-	1	1.2	1	1.0	
Site of origin of primary tumor							
Favorable site	1	5.6	19	22.4	20	19.4	0.1017
Unfavorable site	17	94.4	66	77.6	83	80.6	
Fusion status							
PAX3-FOXO1 positive	-	-	31	36.5	31	30.1	
PAX7-FOXO1 positive	-	-	8	9.4	8	7.8	
FOXO1 positive	-	-	17	20.0	17	16.5	
FOXO1 negative	-	-	28	32.9	28	27.2	
Sample inadequate	-	-	1	1.2	1	0.9	
Not analyzed	18	100.0	-	-	18	17.5	

Abbreviations: aRMS, alveolar rhabdomyosarcoma; FOXO1, forkhead box protein O1; IRS, Intergroup Rhabdomyosarcoma Study; PAX3, paired box 3; PAX7, paired box 7.

accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines (European Union Drug Regulating Authorities Clinical Trials EUDRACT No. 2005-000217-35).

RESULTS

Patient and Tumor Characteristics

From December 2005 to December 2013, a total of 103 patients with aRMS/N1 were included, accounting for 8.1% of all patients (1272 patients) enrolled in the EpSSG RMS2005 protocol. *PAX3/7-FOXO1* fusion was analyzed in 85 patients (82.5%). *FOXO1* gene disruption was detected by FISH or RT-PCR in 56 patients, 31 of whom had *PAX3-FOXO1*, 8 of whom had *PAX7-FOXO1*, and 17 of whom were *FOXO1* positive with an unknown gene partner. Twenty-eight patients had fusion-negative tumors and 1 sample was inadequate for analysis. Molecular study was not performed in 18 patients.

The clinical characteristics of the entire cohort (Table 1) demonstrated a predominance of unfavorable prognostic factors: 90% of patients had IRS group III tumors, 81% of tumors were located at unfavorable sites, 77% of tumors measured > 5 cm, invasive (classified as T2) tumors represented approximately 63% of all cases, and approximately 50% of patients were aged >10 years. No significant differences were found with regard to the prognostic factors considered in the current study between patients with or without biologic data, with the exception of the predominance of age ≥10 years in the group without molecular study ($P = .0339$). For this reason, inferential statistical analyses were performed in patients with available biological data.

Treatment

Chemotherapy

Of the 103 enrolled patients, 73 received chemotherapy as per protocol and 30 received treatment with modifications. Of these 30 patients, 19 had interrupted chemotherapy before completing treatment (18 because of progressive disease or disease recurrence and the parents of 1 patient refused to continue treatment) and in 11 patients chemotherapy was modified because of (CTCAE v4.03) toxicity in 2 patients (septic shock and hemorrhagic cystitis, respectively), a lack of tumor response in 2 patients, and by the attending physician's decision in 7 patients. All patients presented with at least 1 episode of

TABLE 2. Association Between Potential Prognostic Factors and Outcome in Patients With Fusion Status Analyzed

		EFS			OS		
	No. of Patients	Failed	5-Year (95% CI)	*P*	Failed	5-Year (95% CI)	*P*
Age							
<10 y	47	18	60.4 (44.7-73.0)	.0596	16	60.6 (43.4-74.0)	.0797
≥10 y	37	22	44.0 (27.3-59.5)		19	47.9 (30.2-63.6)	
Tumor size							
≤5 cm	20	7	64.3 (39.3-81.2)	.3475	7	59.8 (32.9-78.8)	.6395
>5 cm	63	33	49.9 (36.8-61.6)		28	52.8 (38.6-65.1)	
Tumor invasiveness (T classification)							
T1	33	10	67.3 (47.3-81.1)	.0137	7	71.5 (48.6-85.5)	.0040
T2	51	30	44.8 (30.8-57.8)		28	45.2 (30.8-58.5)	
Fusion status							
Positive	56	33	43.0 (29.5-55.7)	.0101	28	45.5 (30.8-59.2)	.0548
Negative	28	7	74.4 (53.6-87.0)		7	73.7 (52.4-86.6)	
IRS group							
II	9	1	88.9 (43.3-98.4)	.0367	1	87.5 (38.7-98.1)	.0533
III	75	39	49.0 (37.0-60.0)		34	51.0 (38.1-62.6)	
Site of primary tumor							
Favorable site	19	4	75.7 (46.9-90.3)	.0177	3	81.2 (51.9-93.6)	.0293
Unfavorable site	65	36	46.9 (34.2-58.5)		32	48.2 (34.7-60.4)	

Abbreviations: 95% CI, 95% confidence interval; EFS, event-free survival; IRS, Intergroup Rhabdomyosarcoma Study; OS, overall survival.

grade 3 to 4 hematologic toxicity. The most frequent nonhematologic toxicity was gastrointestinal (mucositis) and neurologic (peripheral neuropathy and ileus) (see Supporting Table 1).

Surgery

Ten patients (10%) underwent primary surgery: 6 in IRS group IIb (primary complete resection without microscopic residual disease and lymph node involvement) and 4 in IRS group IIc (primary complete resection with microscopic residual disease and lymph node involvement). A total of 48 patients underwent secondary surgery (resection of the primary tumor in 29 patients, combined resection of the tumor and lymph nodes in 15 patients [1 bilateral lymphadenectomy, 7 unilateral lymphadenectomies, and 7 biopsies], and surgery to the lymph nodes alone in 4 patients [2 biopsies and 2 unilateral lymphadenectomies]). Among the 44 patients who underwent delayed surgical resection of the primary tumor, complete local resection (R0) was performed in 29 patients, with microscopic residual disease (R1) noted in 8 patients, macroscopic residual disease (R2) noted in 4 patients, and no residual tumor noted in 3 patients.

Radiotherapy

Overall, 92 of 103 patients (89.3%) were irradiated. RT was not administered because of progressive disease in 4 patients, amputation in 2 patients, parental refusal in 2 patients, and physician decision in 3 patients. Eight patients received irradiation to the primary tumor area alone, 81 to the primary tumor and lymph nodes, and 3 to lymph nodes alone (2 patients after limb amputation and 1 with a completely resected primary tumor at the time of diagnosis). The median dose to the primary tumor for the overall population was 50.4 Gy (range, 36.0-59.4 Gy) and that to the lymph nodes was 41.4 Gy (range, 24.0-54.4 Gy). Fifteen of 103 patients were aged ≤3 years: 11 received RT and 4 did not receive RT because of parent refusal in 1 patient, physician decision in 1 patient, and tumor progression before the initiation of RT in 2 patients.

Outcome

With a median follow-up of 64.9 months (range, 19.8-116.3 months), 52 patients developed an event and 47 died of disease. Seven patients had refractory disease (no response or disease progression at week 9) and presented with early disease progression (median time to disease progression of 6.2 months [range, 2.1-9.7 months]), 2 patients developed secondary neoplasms, and 43 patients developed disease recurrence. The site of the first recurrence was local in 10 patients, lymph node in 6 patients, and locoregional in 2 patients. Seventeen patients had distant disease recurrence and 8 patients had combined disease recurrence. Local and locoregional events (18 of 43 patients) accounted for approximately 42% of the cases of disease recurrence and lymph node recurrences were present in 13 patients as the first event (33%). The median

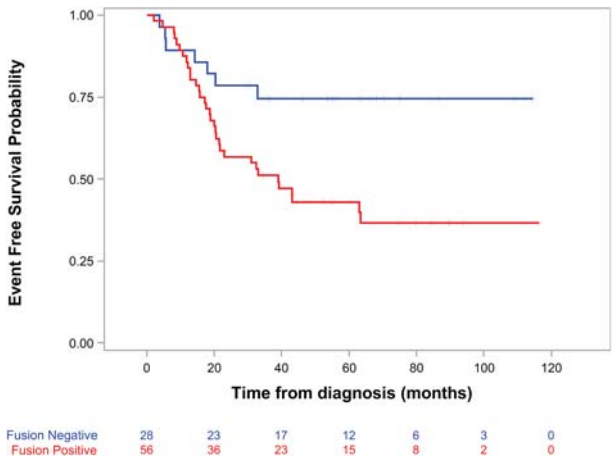

Figure 1. Kaplan-Meier curves representing 5-year event-free survival (EFS) by fusion status. The EFS rate for patients with fusion-positive tumors was 43% compared with 74.4% for those with fusion-negative tumors ($P = .01$).

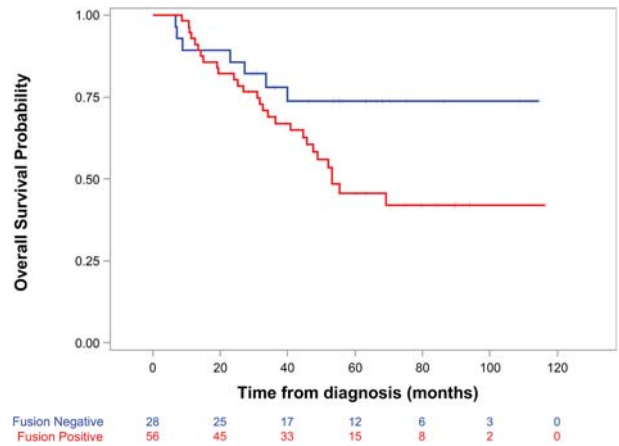

Figure 2. Kaplan-Meier curves representing 5-year overall survival (OS) by fusion status. The OS rate for patients with fusion-positive tumors was 45.5% compared with 74.7% for those with fusion-negative tumors ($P = .05$).

time from diagnosis to disease recurrence was 16.4 months (range, 2.1-63.5 months). At the time of last follow-up, among the 5 patients surviving tumor recurrence, 3 were alive with disease and 2 were in complete response after second-line chemotherapy and RT. The 5-year EFS rate for the entire population was 50.1% (95% CI, 39.8%-59.5%) and the 5-year OS rate was 50.6% (95% CI, 39.7%-60.5%). The median time from first event to death was 8.8 months (range, 0-41.0 months). In the univariate analysis performed in the group of patients for whom fusion status data were available (Table 2), the following factors were found to be associated with an increased risk of disease recurrence or death: unfavorable primary site, invasive tumor (T2 classification), the presence of the *FOXO1* translocation, and classification into IRS group III. Significant variables ($P < .25$) emerged from univariate analysis (patient age at the time of diagnosis, primary tumor site, tumor invasiveness, fusion status, and IRS group) and were included in the Cox model. Only fusion gene status and tumor invasiveness remained as independent prognostic factors for the risk of disease recurrence. Fusion-positive aRMS was associated with EFS with an HR of 2.6 (95% CI, 1.1-5.9; $P = .0226$) and tumor invasiveness (T2 classification) was associated with an HR of 2.2 (95% CI, 1.1-4.6; $P = .0296$). Fusion gene status and tumor invasiveness also remained as independent prognostic factors for the risk of death with an HR associated with fusion-positive tumors of 2.5 (95% CI, 1.1-5.6; $P = .0300$) and an HR related to tumor invasiveness (T2 classification) of 2.2 (95% CI, 1.1-4.6; $P = .0298$). The 5-year EFS rate in patients with fusion-positive tumors was 43.0% (29.6%-55.7%) compared with 74.4% (53.6%-86.9%) in those with fusion-negative tumors ($P = .0101$) (Fig. 1). The 5-year OS rate for patients with fusion-positive tumors was 45.5% (95% CI, 30.8%-59.2%) compared with 74.7% (95% CI, 52.4-86.6) for patients with fusion-negative tumors ($P = .0548$) (Fig. 2).

DISCUSSION

The results of the current study provide evidence of the prognostic impact of fusion status and tumor invasiveness in patients with aRMS and lymph node involvement. Results from previous European and North American cooperative studies have demonstrated very poor survival in patients with aRMS and lymph node involvement, who account for up to 10% of all patients with RMS. In the CWS/RMS86 study, the 3-year EFS rate was 28% and the OS rate was 29%.[18] Results in the SIOP experience were only slightly better, with a 5-year EFS rate of 39% in the SIOP MMT84 study,[19] which is comparable to that of stage IV disease.

The impact of lymph node involvement on prognosis in patients with RMS remains a matter of controversy. Rodary et al[20] evaluated a cohort of 951 international patients with nonmetastatic RMS and identified tumor invasiveness, tumor size, primary tumor site, and N1 disease as prognostic factors. Similarly, in their analysis of patients with nonmetastatic RMS enrolled in American IRS protocols, Meza et al[10] demonstrated that only stage of disease and IRS group were significantly associated with EFS for the majority of patients with aRMS. However, for patients in group III with aRMS, N1 disease was

associated with poorer EFS and OS. These observations influenced the development of the current EpSSG treatment protocol, which assigned patients with aRMS of N1, but not embryonal N1 RMS, to the very high-risk group, for whom a more intensive treatment was recommended.[21]

Rodeberg et al[22] investigated the contribution of regional lymph node disease to the prognosis of patients enrolled in the IRS-IV study. They included 125 patients with localized RMS and lymph node involvement. Patients with alveolar histology and positive lymph nodes were found to have significantly worse 5-year failure-free survival compared with those with alveolar histology without lymph node involvement (43% and 73%, respectively). Moreover, in patients with alveolar histology and N1 disease, outcomes were more similar to those of patients with solitary metastatic disease compared with patients with N0 disease. These results are consistent with the results of the current study. The main difference between the aforementioned study and the current report is that the former included both alveolar and embryonal tumors with lymph node involvement; however, as in the current study, patients with tumors located at unfavorable sites, those with disease at advanced stages, and those with large and invasive tumors were predominant. All these characteristics have been associated with an increased risk of distant metastatic disease.[3,5,23,24] Conversely, involvement of regional lymph nodes in patients with embryonal tumors did not prove to have any negative effect on outcome in the study by Rodeberg et al[22] or in the more recent report by Rogers et al.[25] This could be due at least in part to the intensified treatment with RT and chemotherapy administered, suggesting that patients with lymph node-positive embryonal tumors can attain equivalent outcomes when given intensified treatment. To the best of our knowledge, the overall outcome of the current study cohort was better than the historical series reported to date. The reasons for the apparent improvement in outcome among these patients could be due in part to better risk stratification, more adequate treatment with intensified chemotherapy, systematic local treatment, and improvements in supportive care.

In the current study, a significant number of patients had tumors that did not respond to initial chemotherapy and these individuals presented with progressive disease shortly after diagnosis, thereby representing 14% of those patients who developed disease recurrence. A recent report from Vaarwerk et al[26] demonstrated the lack of correlation between early radiologic response and outcome in patients enrolled in the MMT95 protocol, even though patients with progressive disease were excluded from the analysis. It must be emphasized that the patients with progressive disease in the current study failed to respond to further treatment and the chance of cure after disease recurrence was very low (5% of the entire cohort), which suggests that patients with refractory disease or disease recurrence could be offered experimental therapy immediately after tumor events. Nevertheless, even with the implementation of combined local therapy with delayed surgery and systematic RT to the primary tumor site and lymph nodes in the current study protocol, locoregional disease recurrences were frequent and accounted for approximately 42% of the initial events. Furthermore, lymph node failures occurred in approximately 33% of the disease recurrences. Some authors have recommend that the in-transit lymphatics be imaged at the time of diagnosis.[27] The involvement of in-transit lymph nodes could be better assessed by performing systematic [^{18}F]fludeoxyglucose (FDG) positron emission tomography-computed tomography at the time of diagnosis, a procedure that was not performed routinely in the cohort of patients in the current study. Moreover, the question of whether in-transit lymph nodes should be irradiated routinely remains unsolved, given the risk of significant toxicity associated with extensive irradiation in pediatric patients.[28,29]

In the current series, we identified some variables found to have prognostic significance on univariate analysis (unfavorable site of tumor origin, tumor invasiveness, *FOXO1* fusion, and IRS group III). However, on multivariate analysis, only tumor invasiveness and the presence of a characteristic fusion gene associated with aRMS resulted in independent predictors of disease recurrence or death. This is consistent with several studies that correlated the presence of a fusion gene with poorer outcome; however, to the best of our knowledge, the real contribution of the presence of *PAX3/7-FOXO1* fusions to the outcome of aRMS remains to be elucidated.[30-32] In the current series, approximately 66% of tumors were fusion positive. These figures are lower than the rate of 70% to 75% reported in the literature, which could be due in part to the fact that fusions involving *PAX3* with partners other than *FOXO1* were missed in the current analysis.[33] We will attempt to avoid these false-negative results in the future EpSSG protocol: in an alveolar tumor that is negative for *PAX3/7-FOXO1* by RT-PCR and for *FOXO1* rearrangement by FISH, additional FISH assessments for the disruption of *PAX3* will be made.

In the current study, fusion status appeared to identify the "real" very high-risk population, thereby

highlighting the importance of performing biologic studies in all patients. We did not attempt to analyze outcome according to the type of fusion because of the limited number of patients with the *PAX7-FOXO1* fusion.

Survival in this newly defined very high-risk group is comparable to results observed in patients with metastatic aRMS treated in the high-risk COG studies D9802 and ARST0431.[34] In those studies, fusion status was not found to be an independent prognostic factor, despite better EFS noted in patients with fusion-negative aRMS. Poorer outcomes for patients with metastatic disease in the COG report were most closely related to other clinical risk factors, including age, primary tumor site, and number of metastatic sites.

The clinical implications of the current study will include a new stratification for patients with aRMS/N1 disease according to fusion status in the future EpSSG RMS study. Patients with fusion-negative N1 tumors will be treated with a strategy similar to that for those with eRMS/N1 disease, with no reduction in treatment intensity. Patients with fusion-positive N1 disease will be treated in the same group as patients with metastatic tumors. For patients with refractory disease or disease recurrence, the EpSSG is working to establish an effective, innovative strategy for the study of new agents and the inclusion of patients in phase 1 and 2 clinical trials.

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CONFLICT OF INTEREST DISCLOSURES

Soledad Gallego has acted in a paid advisory role for Loxo Oncology (one conference) and Clinigen Group (one conference) for work performed outside of the current study. Christophe Bergeron is supported by the Association Leon Berard Enfant Cancereux (ALBEC). Julia Chisholm has acted as a paid consultant for F. Hoffman La Roche for work performed outside of the current study and she and Henry Mandeville have received funding from the National Institute for Health Research (NIHR) Biomedical Research Centre at the Royal Marsden National Health Service Foundation Trust and the Institute of Cancer Research for work performed as part of the current study. Gianni Bisogno has acted in a paid advisory role for Loxo Oncology (one conference) and Clinigen Group (one conference) and as a paid member of the Speakers' Bureau for Merck and Company and received a grant from INDENA S.p.A. for work performed outside of the current study.

AUTHOR CONTRIBUTIONS

Soledad Gallego: Conception and design, collection and assembly of data, and data analysis and interpretation. **Ilaria Zanetti**: Data analysis and interpretation. **Gian Luca de Salvo**: Conception and design and data analysis and interpretation. **Gianni Bisogno**: Conception and design, collection and assembly of data, and data analysis and interpretation. **All authors**: Article writing and final approval of article.

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Original research

Conservative strategy in infantile fibrosarcoma is possible: The European paediatric Soft tissue sarcoma Study Group experience

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KEYWORDS
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Chemotherapy;
ETV6-NTRK3 transcript

Abstract ***Background:*** Infantile fibrosarcoma (IFS) is a very rare disease occurring in young infants characterised by a high local aggressiveness but overall with a favourable survival. To try to reduce the total burden of therapy, the European pediatric Soft tissue sarcoma Study Group has developed conservative therapeutic recommendations according to initial resectability.

Material and methods: Between 2005 and 2012, children with localised IFS were prospectively registered. Initial surgery was suggested only if possible without mutilation. Patients with initial complete (IRS-group I/R0) or microscopic incomplete (group II/R1) resection had no further therapy. Patients with initial inoperable tumour (group III/R2) received first-line vincristine-actinomycin-D chemotherapy (VA). Delayed conservative surgery was planned after tumour reduction. Aggressive local therapy (mutilating surgery or external radiotherapy) was discouraged.

Results: A total of 50 infants (median age 1.4 months), were included in the study. ETV6-NTRK3 transcript was present in 87.2% of patients where investigation was performed. According to initial surgery, 11 patients were classified as group I, 8 as group II and 31 as group III. VA chemotherapy was first delivered to 25 children with IRS-III/R2 and one with IRS-II/R1 disease. Response rate to VA was 68.0%. Mutilating surgery was only performed in three cases. After a median follow-up of 4.7 years (range 1.9–9.0), 3-year event-free survival and overall survival were respectively 84.0% (95% confidence interval [CI] 70.5–91.7) and 94.0% (95% CI 82.5–98.0).

Conclusions: Conservative therapy is possible in IFS as only three children required mutilating surgery, and alkylating or anthracycline based chemotherapy was avoided in 71.0% of patients needing chemotherapy. VA regimen should be first line therapy in order to reduce long term effects.

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1. Introduction

Although infantile fibrosarcoma (IFS) is a rare tumour, it is the commonest soft tissue sarcoma in children less than 1 year of age. IFS is currently classified as a soft tissue tumour of intermediate malignancy characterised by a quite specific t(12;15)(p13;q25) translocation coding for a ETV6-NTRK3 gene fusion [1–3]. It arises below the age of 2–5 years with survival rates between 80 and 100% [1,4,5]. It often presents with initial rapid growth, sometimes with indolent evolution and metastatic spread is uncommon (1–13%). Local recurrence may occur after initial conservative surgery (17–43%), the latter being the mainstay of treatment, aiming for a conservative resection. However, IFS may present with locally advanced disease and surgery maybe mutilating or cause functional damage [4,5]. Since IFS is a chemosensitive tumour, chemotherapy may play a major role in the treatment strategy [1,6,7]. Recently, the VA regimen (vincristine-actinomycin-D), has confirmed its efficacy and allows important tumour reduction [1]. The International Society of Pediatric Oncology–Malignant Mesenchymal Tumour Committee and the Associazione Italiana Ematologia Oncologia Pediatrica–Soft Tissue Sarcoma Committee (previously called the Italian Cooperative Group) founded the European-paediatric-Soft-tissue-Sarcoma-Study Group (EpSSG) in 2005. The group developed treatment guidelines for IFS, with the major goal to make uniform the treatment of IFS patients across Europe, according to a conservative approach based on non-mutilating surgery and alkylating-anthracycline-free chemotherapy (EpSSG non-rhabdomyosarcoma soft tissue sarcomas [NRSTS] 2005 study – European Union Drug Regulating Authorities Clinical Trial No. 2005-001139-31) This present paper reports the results of a prospective cohort of IFS patients treated between 2005 and 2012 aiming to propose a conservative strategy in this disease.

2. Patients and methods

2.1. Study population

All infants aged from birth to 24 months with localised IFS were prospectively registered in the EpSSG database using a web-based system, from October 2005 to 30th June 2012. Patients were classified according specific tumour sites [8]. Clinical staging was defined according to the tumour node metastases system: T1 or T2 according to the invasion of contiguous organs; N0/N1, and M0/M1 according to the presence of lymph node or distant metastases [8]. Lymph node involvement was evaluated clinically or by imaging and confirmed when necessary by cytological or histological biopsy. The status of resection margins was classified according to the UICC-R classification and the Intergroup

Rhabdomyosarcoma Staging (IRS) system which is generally used for primary surgery in paediatric rhabdomyosarcomas [9]. UICC-R R0 or IRS group I correspond to complete tumour resection with histologically free margins, UICC-R R1 or IRS II correspond to macroscopic resection, but invaded margins on histology, UICC-R R2 or IRS III correspond to macroscopic residual tumour after surgery (III b) or biopsy (III a). Patients with metastatic tumours were excluded from the analysis.

Cytogenetic and molecular evaluation to identify the presence of ETV6-NTRK3 transcript derived by the specific translocation by FISH and RT-PCR were recommended to confirm the diagnosis [10]. Where there was doubt, tumours were prospectively reviewed at the time of diagnosis by national and/or international panel of pathologists [11,12]. Exclusion criteria were: histological review did not confirm IFS diagnosis (n = 3); tumours negative for the ETV6-NTRK3 transcript or not tested in the absence of pathologic panel review (n = 4) (Fig. 1). Institutional ethics board approval was obtained for all participating centres according to the rules established in Europe. Written consent for treatment and the use of data were obtained from parents or guardians according to local research ethics requirements.

2.2. Treatment

Primary surgery after initial biopsy was recommended only when *en bloc* resection, removing the tumour through normal tissue with clear margins, might be achieved without significant long-term functional or cosmetic impairment. In the other cases, a biopsy was required followed by chemotherapy and, if necessary, delayed surgery. No adjuvant chemotherapy was recommended if resection was complete or microscopically-incomplete (IRS group-I/R0 or II/R1). The VA regimen was the treatment of choice in patients with unresectable disease (IRS group III/R2), with the exception of patients with congenital tumours (age <3 months at diagnosis), for which an optional 'wait and see' strategy was considered to evaluate the possibility of spontaneous regression or time to facilitate subsequent surgery. VA chemotherapy was continued, in a responsive tumour, until tumour resectability was possible. If the tumour shrinkage was not sufficient to permit conservative surgery, ifosfamide (IVA regimen) or cyclophosphamide (VAC regimen) was added (Fig. 2). Where there was no response to VA, or tumour progression, ifosfamide-doxorubicin (ID) chemotherapy was recommended. Mutilating surgery and external radiotherapy was strongly discouraged.

Additional dose reductions were applied for infants <8 kg and <6 months (30% reduction), and for newborns <5 kg and <3 months (50%). Moreover, the initial doses were delivered at 50%, progressively increasing to 75% and 100% to verify overall tolerance in infants, with specific attention to neurologic and hepatic toxicity, particularly constipation and veno-occlusive disease (VOD). No alkylating agent was administered before 1 month and no anthracycline before 3 months of age.

2.3. Response assessment

In patients with measurable disease, response to chemotherapy was assessed after three cycles of chemotherapy by assessment of radiologically-identified tumour volume reduction: i.e. complete response (CR)

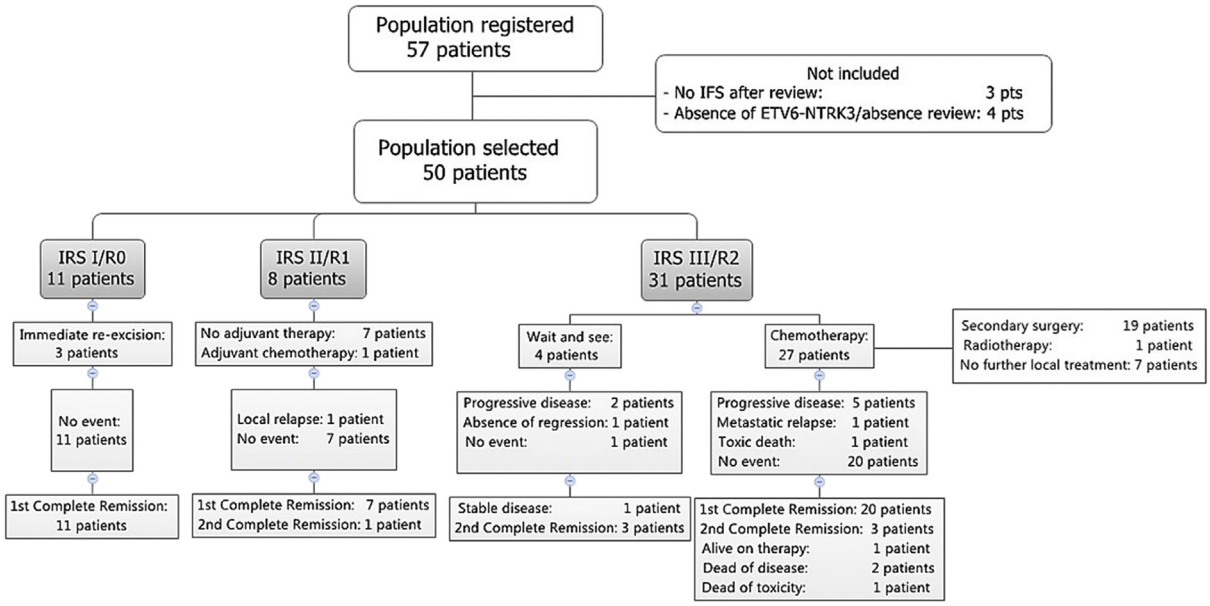

Fig. 1. Patient's charts. Abbreviations: IFS, infantile fibrosarcoma; IRS-I/R0, complete exeresis; IRS-II/R1, microscopic residue; IRS-III/R2, macroscopic residue; pts, patients.

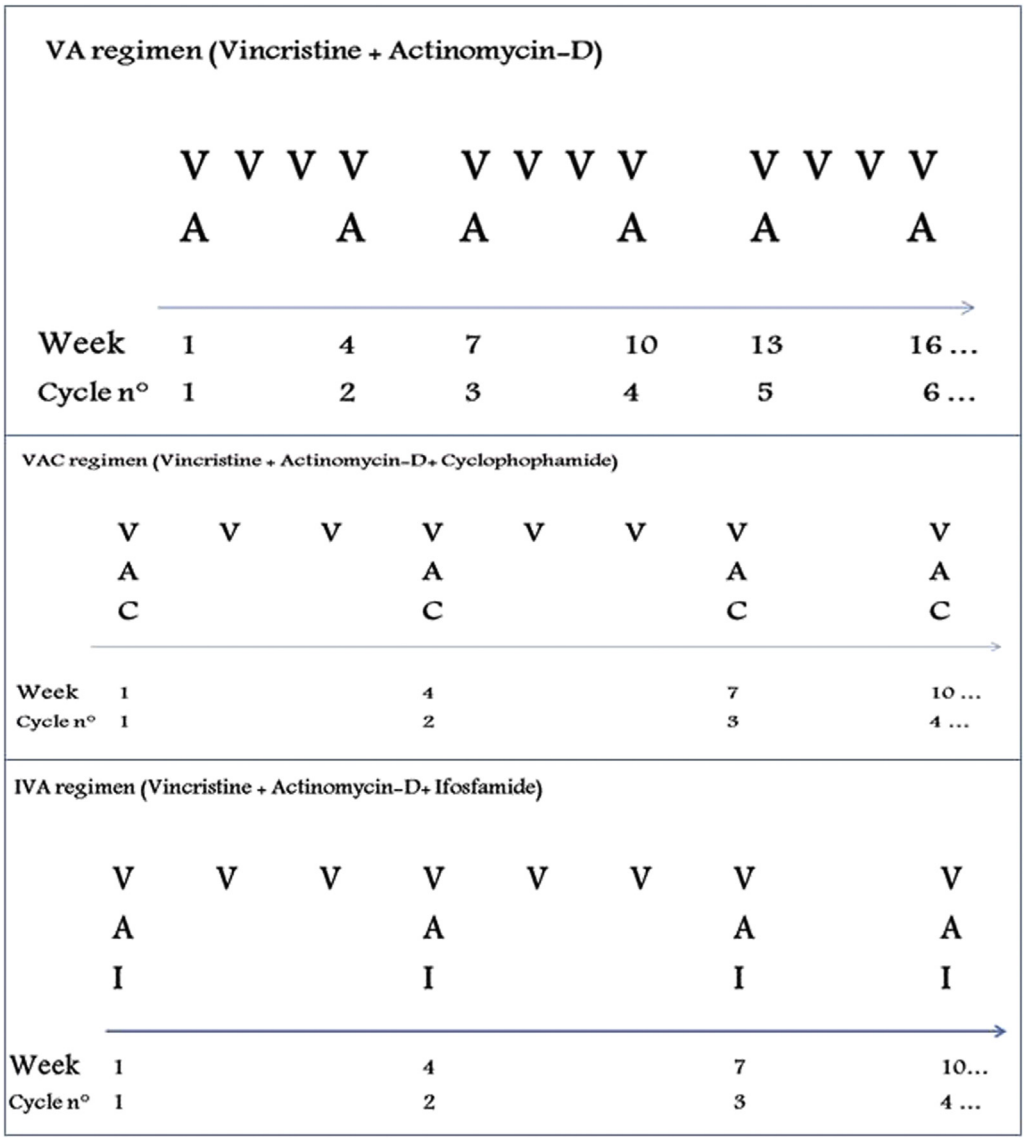

Fig. 2. Chemotherapy schedules. **Chemotherapy dosages**: Vincristine and actinomycin-D: 50 μg/kg/injection; if age >12 months and weight >10 kg: 1.5 mg/m^2. Endoxan®-cyclophosphamide: 50 mg/kg; if age >12 months and weight >10 kg: 1.5 g/m^2. Holoxan®-ifosfamide: 100 mg/kg × 2 d for patients >3 months and 5–10 kg; of age >12 months and weight >10 kg: 3 g/m^2 × 2 d. See text for further reductions in young children.

= complete disappearance of visible tumour with no residual disease; major partial response (PR ≥2/3) = volume response 66–99%; minor PR (<2/3) = volume response 34–65%; stable disease (SD) ≤ 33% reduction in tumour volume; progressive disease (PD) = more than 40% increase in the sum of the volumes of all measurable lesions, or the appearance of new lesions [13]. Response rate to specific regimen of chemotherapy was considered as follows: (CR+ PR ≥2/3+ PR <2/3).

2.4. Statistical methods

Data were analysed by the International Data Center (Istituto Oncologico Veneto I.R.C.C.S., Padua; Italy), considering information within the Remote Data Entry system as at May 2015. Outcome was defined as overall survival (OS) and event-free survival (EFS). The definition of OS was measured from the date of diagnosis to death from any cause. Events were defined for EFS as progression during chemotherapy, relapse after CR or death from any cause. Local control was defined as disappearance of all radiological signs of disease at the site of the primary or stable residual radiographic images for at least 6 months after completion of treatment. Survival curves were calculated by the Kaplan-Meier method [14]. The 3-year EFS and OS were reported along with their 95% confidence intervals (CI).

3. Results

A total of 50 cases with a diagnosis of IFS and age <2 years were considered during the study period. They represent 6.5% of all registered patients with NRSTS and 30.1% of those aged less than 2 years included in the NRSTS EpSSG database. Four older patients (>2 years) were registered during the same time in this database but their tumours did not manifest the specific transcript and were not included in the analysis. Overall clinical characteristics of the population are indicated in Table 1. Most tumours were not associated with specific congenital abnormalities (95.9%). Median age at diagnosis was 1.43 months (range: 0.03–18.73). The diagnosis was made before birth or during the first month of life for 40.0% of the patients and in 68.0% of the cases before the age of 3 months (so called 'congenital forms'). Tumours occurred mainly in the limbs (54.0%), with more than half ≥5 cm at diagnosis and none had lymph node spread. Histological local diagnosis was confirmed by national and/or international histology review in 39 and 15 cases respectively. The identification of ETV6-NTRK3 transcript was tested in 39/50 patients: FISH showed the presence of the fusion gene in 9/11 samples, RT-PCR was positive in 19/21 samples, and both tests were positive in 6/7 additional patients. All cases without histology review harboured the ETV6-NTRK3 translocation. In summary, the characteristic biological translocation was identified in 87.2% of tumours.

Table 1
Clinical characteristics of the population.

	Number of patients n = 50	%
Age at diagnosis (months)		
<1	20	40.0
1–3	14	28.0
4–12	12	24.0
>12	4	8.0
Congenital abnormalities associated		
Yes	2	4.0
Ductus arteriosus persistens	*1*	*50.0*
Occipital haemangioma	*1*	*50.0*
No	47	94.0
Missing data	1	2.0
Gender		
Female	18	36.0
Male	32	64.0
Post-surgical tumour staging (IRS)		
Group I (R0)	11	22.0
Group II (R1)	8	16.0
Group III a (biopsy) (R2)	27	54.0
Group III b (incomplete surgery) (R2)	4	8.0
Primary tumour invasiveness (T)		
T1 – Localised to the organ or tissue of origin	33	66.0
T2 – Extending beyond the tissue or organ of origin	17	34.0
Tumour size		
a:≤5 cm	23	46.0
b:>5 cm	27	54.0
Regional lymph node involvement		
N0-No evidence of lymph node involvement	50	100
Site of origin of primary tumour		
Extremities	27	54.0
Axial sites	14	28.0
Abdomen	*7*	
Paraspinal	*2*	
Retroperitoneal	*2*	
Thorax	*2*	
Trunk	*1*	
Non-parameningeal head and neck	4	8.0
Parameningeal	3	6.0
Genito-urinary non Bladder Prostate	2	4.0
Kidney	*2*	

3.1. Treatment according to IRS group

According to initial surgery, eleven patients were classified as IRS-group I/R0, eight as IRS-group II/R1, thirty one as IRS-group III/R2, four after resection with macroscopic residual tumour and twenty seven after biopsy (Fig. 1).

IRS I-II-group (n = 19): three out of 19 patients underwent primary re-excision of the tumour. No adjuvant chemotherapy was given according to the guideline recommendations in all but one case (a ruptured atypical hypercellular mesoblastic nephroma primary, IRS-group II/R1) that received 6 months of VA (treating physician's decision) with additional vincristine-cyclophosphamide for 2 months due to mild hepatic toxicity. Surgery comprised of wide tumour excision (18 cases), associated with a partial colectomy (three cases) or unilateral nephrectomy (two cases) and a limited chest wall excision (one case). One local relapse occurred in this group: a 17-d-old baby with a right wrist IFS who suffered a local relapse 2.5 months after microscopic incomplete surgery and then underwent radical surgery. He remains in 2nd CR 4 years after diagnosis. All 19 patients were alive in remission at the time of the analysis.

IRS III-group (n = 31): Chemotherapy was administered to 27 patients for a median duration of 4.14 months (range: 0.46–12.06). The remaining four had a 'wait and see' strategy. Overall 25 patients started chemotherapy according to the protocol with VA regimen for 14 d to 12 months, median 4.14 months. Six patients then switched chemotherapy to IVA (three cases), VAC (two cases), or ID regimen (one case) either due to SD or PD (three cases), to facilitate surgery (two cases) or for a life threatening scenario (one case). Finally, one patient received the IVA regimen due to an initially incorrect diagnosis and another one received VAC by physician preference due to rapid growth of the tumour after diagnosis.

A wait and see approach was used for four IRS group-III/R2 patients. Among them, three patients needed delayed VA chemotherapy from 2–4 months

after diagnosis, all with response (1 CR, 2 PR>2/3) and are alive in remission at the end of follow up. One is alive with a residual mass after spontaneous tumour reduction (Table 2).

The overall response rate to chemotherapy was 62.9% (17/27 evaluable patients) and 68.0% specifically to VA regimen (17/25 cases). Tolerance of chemotherapy was manageable overall but seven cases had specific grade III–IV toxicity: three reversible VOD, one peripheral neurotoxicity with ptosis, one haematological grade IV neutropenia with grade III anaemia, one haemorrhage in the tumour during progression. A 1-month old patient received by error an overdose (100 fold) of actinomycin-D and died despite supportive care.

Delayed tumour surgery was performed after a median of 4.9 months (range: 1.2–20.5) from diagnosis in 19 cases, with a wide excision in 13 patients including a conservative parotidectomy (one case), a limited perineal excision (one case), and nephrectomy with adrenalectomy (one case). Limb amputation was performed for two children and an exenteration in one patient. Overall, resection was complete in 14 cases, with microscopic residual in four cases and a macroscopically incomplete resection in one patient. No further surgery was done for 11 IRS-III/R2 patients. In seven cases, this was due to physician's decision (despite a residual images following chemotherapy in four cases; after an initial wait and see strategy in three cases), after histological remission of a residual mass confirmed by biopsy (two cases), and a clinical complete remission after chemotherapy (one case).

3.2. Total burden of therapy

Among the 50 cases, 40 (80.0%) had tumour surgery: resection alone in 19 cases and associated with chemotherapy in 21 cases. Surgery was mostly conservative (37 cases) whereas three needed mutilating surgery. Only one patient with a progressive orbital parameningeal IFS despite VA than VAC regimens received proton radiotherapy at 54 Gy after an orbital exenteration. Two other patients had limb amputation (finger, hand). Overall, among the 47 survivors, chemotherapy was delivered in 29 cases (61.7%) and comprised a VA regimen alone (22 cases), with additional alkylating agents (six cases) and/or anthracycline drug (one case).

3.3. Congenital cases

Among the 34 infants with congenital IFS, 59.0% were discovered antenatally or before 1 month of age. The site was the limbs (47.1%), 'other' sites (35.3%), head-and-neck (11.8%) and genito-urinary (5.9%).

3.4. Outcome

At the time of analysis, 35 patients are in first complete remission (CR), one is alive after 1st line chemotherapy; one is alive with a residual mass after therapy, seven are in 2nd or greater CR off therapy, three have died and three are lost to follow-up in CR (Fig. 1). Ten patients had a tumour event, nine initially classified as IRS group III/R2 and one as IRS group II/R1: seven tumours progressed, one patient experienced a metastatic relapse, one had a local relapse and one patient died due to toxicity. Among the 10 cases with tumour events, eight had tumours with ETV6-NTRK3 transcript, one without and in one case the analysis had not been performed. Tumour progression occurred in two cases after a wait and see strategy (Table 2), after VAC-IVA/ID regimens (two cases with refractory disease responsible for patients' death), and after the VA regimen (three cases were treated with VAC and surgery, surgery alone and ID regimen, and are in subsequent CR). One patient developed lung metastases 2.5 years after a head and neck tumour initially unresponsive to VA but

Table 2
IRS III/R2 patients with a 'wait and see' strategy.

	Patient no 1	Patient no 2	Patient no 3	Patient no 4
Age at diagnosis	10 d	41 d	68 d	11 months
ETV6-NTRK3 transcript	Presence	Presence	Presence	Presence
Site of primary tumour	Foot	Shoulder	Retroperitoneal	Tight
Invasiveness	T1	T2	T1	T1
Tumour size	≤5 cm	>5 cm	>5 cm	≤5 cm
Time from diagnosis to start of CT	4 months	–	3 months	2 months
Reason for treatment	Progressive disease	–	Progressive disease	Absence of regression
Therapy	CT (VA regimen for 5 months)	–	CT (VA regimen for 3 months)	CT (VA regimen for 5 months) + HCR after delayed surgery
Status	Alive in CR off therapy	Alive with tumour decreased from diagnosis	Alive in CR off therapy	Alive in 1st CR off therapy
Time from diagnosis to last FUP	5 years and 7 months	2 years and 11 months	3 years	7 years and 10 months

Abbreviations: CT chemotherapy, HCR histologic complete response; CR complete remission; VA vincristine-actinomycin-D, FUP follow-up.

responding to subsequent ID chemotherapy, (allowing a R1 resection). At the time of the report, this patient was alive in secondary remission after second-line chemotherapy and pulmonary metastasectomy. One patient died due to an overdosage of chemotherapy. Two patients died from disease. The three patients that received mutilating surgery are alive and in continuing complete remission off therapy. After a median follow-up of 4.7 years (range 1.9–9.0), 3-year EFS and OS were respectively 84.0% (95% CI 70.5–91.7) and 94.0% (95% CI 82.5–98.0) (Fig. 3).

4. Discussion

This study demonstrates that a conservative treatment approach is feasible in young children with IFS without jeopardising survival. Despite many having large tumours at diagnosis, mutilating surgery was only required in three cases and alkylating-anthracycline-free chemotherapy sufficient to achieve cure in 74.2% of patients requiring chemotherapy. Our experience also confirms that prospective multi-institutional trials are possible even in very rare tumours in children at an European level [15]. The very good compliance with treatment guidelines within the different European countries involved, e.g. 94.7% of IRS I-II group patients were treated with surgery alone, and 93.3% of IRS group III patients received the VA regimen as first line therapy as recommended, shows that the goal to standardise the IFS treatment was achieved.

This series confirms some of the general clinical characteristics of IFS as a rare disease occurring in very young patients (median age 1.43 months), predominantly in males (64.0%), and mainly in limbs (54.0%) [16,17]. According to some authors, IFS can be either a histological or a biological defined entity [4,11,18]. This series reported that the ETV6-NTRK3 fusion gene documented by FISH or RT-PCR was present in 87.2% of the patients with IFS where the investigation was performed. This is a helpful tool where there is pathological difficulty in confirming the diagnosis of IFS [10]. Nevertheless, it is important to note that the ETV6-NTRK3 fusion gene is not totally specific to IFS. It has been described in congenital hypercellular mesoblastic nephroma, mammary analogue secretory carcinoma of salivary glands and of the breast, and in some leukaemias [19]. The definition of the 'infantile' nature of fibrosarcoma is not precise in the literature and an age limit up to 2 years is used by most authors [16,17,20,21]. Even if some rare series consider patients with IFS up to 3 years, we focused on the population of very young children (≤2 years of age at diagnosis) for whom the consequences of treatment (chemotherapy, radical surgery and radiotherapy) are a major factor guiding treatment decisions, and also to be consistent with other analyses [1,6,22].

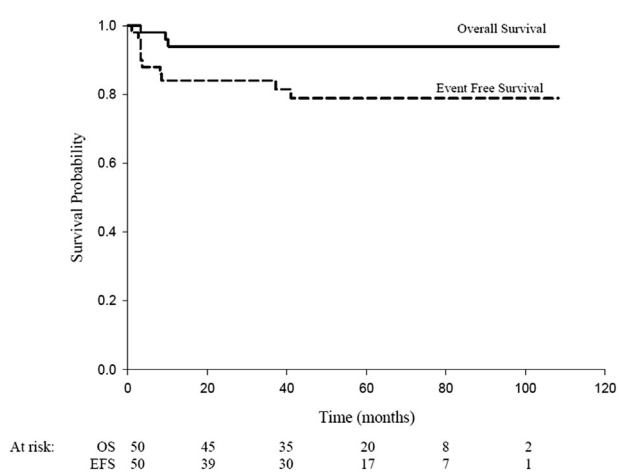

Fig. 3. Event-free survival and overall of the entire population. Abbreviations: OS, overall survival; EFS, event-free survival.

Other studies previously reported the very good OS of children with IFS, and emphasised the challenge of tumour resectability without anatomical or functional damage. Even if surgery should still be seen as the cornerstone of therapy in this tumour, our experience highlighted that the use of chemotherapy may also play a critical role in large diffuse inoperable tumours. Initial grossly tumour resection (IRS-I/II–R0/1) was only possible in 38.0% of patients but tumour shrinkage achieved with chemotherapy in the majority of initially unresected tumours allowed a secondary conservative surgical approach in the majority. It is clear, however, that postoperative chemotherapy is not necessary after a delayed complete macroscopic tumour resection (IRSI/II-R0/1) or total necrosis. Similarly, adjuvant chemotherapy was unnecessary for IRS group I/R0 patients but also for IRS group II/R1. In this cohort, only one local recurrence occurred out of 19 patients, and was successfully treated with further surgery. Nevertheless, the overall consensus should be to try to avoid incomplete surgery with macroscopic residue.

The VA regimen, a combination that does not contain alkylating agents or anthracyclines, appears to be very active in IFS. Acute toxicity was not negligible but despite three mild episodes of VOD and one toxic death (associated with a dose error) we believe that VA is more advantageous compared with VAC chemotherapy (cyclophosphamide) or anthracycline containing regimens, as it reduces the gonadal and mutagenic toxicity of cyclophosphamide/ifosfamide and the cardiac toxicity of anthracyclines in very young children, previously used in up to 53–87% of all patients [6,20,23,24]. The optimal duration of preoperative chemotherapy was not defined in our protocol and still needs to be clarified.

Previously it was unclear whether it is possible to avoid delayed surgery in IRS-group III/R2 patients, after successful use of neoadjuvant chemotherapy with

radiological complete remission. In our experience, 35.4% (11/31) of IRS-III/R2 patients did not need delayed resection due to a radiologic CR or VGPR after neoadjuvant chemotherapy, and we therefore recommend this approach.

The possibility of spontaneous regression in IFS has already been reported [25–27]. The observation that one patient in our series showed a spontaneous tumour shrinkage supports a 'wait and see' strategy especially in very young patients, i.e. patients <3 months with a non-resectable primary in a non-threatening situation, in whom tolerance to chemotherapy may be poor. This approach may be extended to older infants, if strict follow-up could be ensured. If progression does occur, then neoadjuvant chemotherapy with VA should be started.

The small number of relapses in our cohort does not allow further analysis of prognostic factors and subsequent risk-stratification. A recent epidemiological retrospective study among a large cohort of 224 children ≤2 years with IFS did not show any significant survival difference according to various risk factors such as margin status, nodal involvement, tumour size or treatment modalities [17].

In conclusion, this study highlights the importance of paediatric international cooperation in developing prospective studies for very rare childhood tumours. Due to the rarity of this tumour all medical decisions should be shared through multidisciplinary discussions at a regional, national or international level [15]. This should allow a conservative treatment approach where feasible in young children with IFS without jeopardising survival.

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Conflict of interest statement

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organisations within that could inappropriately influence (bias) their work.

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Clinical Trial

Outcome of extracranial malignant rhabdoid tumours in children registered in the European Paediatric Soft Tissue Sarcoma Study Group Non-Rhabdomyosarcoma Soft Tissue Sarcoma 2005 Study—EpSSG NRSTS 2005

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KEYWORDS
Malignant rhabdoid tumour;
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Prospective registry;
Survival;
Prognostic factors

Abstract ***Background:*** Extracranial malignant rhabdoid tumours (MRT) are rare lethal childhood cancers that often occur in infants and have a characteristic genetic mutation in the *SMARCB1* gene. The European Paediatric Soft Tissue Sarcoma Study Group (EpSSG) conducted a multinational prospective study of registered cases of extracranial MRT to test an intensive multimodal approach of treatment for children with newly diagnosed extracranial MRT.

Methods: Between December 2005 and June 2014, we prospectively registered 100 patients from 12 countries with a diagnosis of MRT tumour at an extracranial site on the EpSSG Non-Rhabdomyosarcoma Soft Tissue Sarcoma 2005 Study (NRSTS 2005). They were all treated on a standard multimodal protocol of surgery, radiotherapy, and chemotherapy over 30 weeks as follows: vincristine, cyclophosphamide, and doxorubicin (VDCy) at weeks 1, 10, 13, 22, and 28; vincristine was also given alone on weeks 2, 3, 11, 12, 14, 15, 23, 24, 29, and 30.

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Cyclophosphamide, carboplatin, and etoposide (Cy*CE) was given at weeks 4, 7, 16, 19, and 25. Radiotherapy was recommended for all primary tumour sites and all sites of metastatic disease.

Results: Forty-three patients completed the protocol treatment. Median follow-up for alive patients of the complete cohort was 44.6 months (range 11.5–84.6). For the whole cohort, the 3-year event-free survival (EFS) was 32.3% (95% confidence interval [CI] 23.2–41.6%) with a 3-year overall survival (OS) of 38.4% (95% CI 28.8–47.9%). For localised disease, the 4-year EFS was 39.3% (95% CI 28.2–50.1%) with a 4-year OS of 40.1% (95% CI 28.4–51.5%). For metastatic disease, the 2-year EFS was 8.7% (95% CI 1.5–24.2%) with a 2-year OS of 13.0% (95% CI 3.3–29.7%). Multivariable analysis disclosed that all patients ≤1 year of age were associated with at higher risk of death (hazard ratio [HR]: 2.6; 95% CI 1.0–6.8; p-value = 0.0094). Risk of death was also related with gender in metastatic patients (HR for males: 2.9, 95% CI 1.0–8.0; p-value = 0.0077).

Conclusions: The EpSSG NRSTS 2005 protocol of intensive therapy can be delivered to extracranial MRT patients, with a possible improvement in outcome. The outcome, however, remains poor for patients who progress or with metastatic disease.
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1. Introduction

Extracranial MRT tumours are rare and often occur in infants with an age standardised incidence ratio of 0.6 per million children in the United Kingdom (UK), 61% of cases in the first of year of life [1]. The vast majority contain a somatic bi-allelic inactivating mutation in the *SMARCB1* gene, which is part of the chromatin remodelling complex SW1/SWF, important in cell cycle control, and functions as a classic tumour suppressor gene [2]. MRT are often described as lethal, with little evidence of improved survival in recent years. In the UK population-based National Registry of Childhood Tumours during 1993 to 2010, the 1-year overall survival (OS) was only 31% [1]. This poor survival is also reflected in the National Wilms' Tumour Study (NWTS) series, and in the United States, Surveillance Epidemiology and End Results (SEER) programme, OS, at 4 years was 23.3% and 33.0%, respectively [3,4]. Given the rarity of extracranial MRT, there is no standard therapeutic pathway, and there has been no randomised or prospective trials examining the role of chemotherapy combinations or, indeed, the addition of new agents. Instead, there have been small retrospective series published either from single institutions or larger series of MRT at single anatomical sites from other site-specific studies, such as NWTS [3,5]. Despite the challenging nature of this tumour and its treatment, two case reports including two and one patients, respectively, with metastatic renal MRT are often cited in view of their successful outcome [6,7]. Based on these reports, the European Paediatric Soft Tissue Sarcoma Study Group (EpSSG) conducted a multinational prospective study of registered cases of extracranial MRT to test an intensive multimodal approach of treatment for children with newly diagnosed extracranial MRT.

2. Methods

2.1. Patients and study design

One hundred patients with a diagnosis of MRT at an extracranial site were registered on the EpSSG Non-Rhabdomyosarcoma Soft Tissue Sarcoma 2005 Study (NRSTS 2005). This was a prospective observational study for all NRSTS patients, with recommended treatment for MRT. The study was conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines. Informed written consent was obtained for all patients/parents. The study was managed via a Web-based system provided by CINECA, an Inter-University Computing Consortium (Casalecchio, Italy).

2.2. Pathological analysis

National and international review by the pathology panel of the histological diagnosis was advised but not considered mandatory. Patients were included if their local histological diagnosis of MRT was supported by immunohistochemistry demonstrating loss of nuclear expression of INI-1 (BAF47 antibody) and/or molecular testing demonstrated deletion of the *SMARCB1* gene [2]. Additional national and/or an international review by the EpSSG panel of pathologists were performed in 64 of the cases.

2.3. Staging and surgery

Following staging investigations, including either computed tomography (CT) or magnetic resonance imaging (MRI) of the primary site, CT scan chest, MRI/CT scan of brain, and for some bone scan and bone marrow assessment, it was recommended for all patients

to undergo surgical resection of primary tumour but if deemed unresectable, biopsy only. The Intergroup Rhabdomyosarcoma Study (IRS) and TNM post-surgical staging was used [8]. Complete resection with no microscopic disease was R0, with microscopic disease was R1, and macroscopic disease was R2.

2.4. Chemotherapy

Following initial surgery or biopsy, the recommended chemotherapy was given over 30 weeks as follows: vincristine, cyclophosphamide, and doxorubicin (VDCy) at weeks 1, 10, 13, 22, and 28; vincristine was also given alone on weeks 2, 3, 11, 12, 14, 15, 23, 24, 29, and 30; cyclophosphamide, carboplatin, and etoposide (Cy*CE) given at weeks 4, 7, 16, 19, and 25 (see Appendix 1 for full dose and schedule plan). Dosages were adapted to infant weight and progressively increased. No details about doses of chemotherapy were collected, but data were available on whether treatment was received and if completed.

2.5. Radiotherapy

Radiotherapy was recommended for all primary tumour sites and all sites of metastatic disease, either following up-front surgery at week 2 or following delayed surgery at week 14. The chemotherapy schedule allowed concomitant radiotherapy. The dose up to a maximum of 50.4 Gy, treatment volume, and fractionation depended on the site of the primary tumour, degree of resection, site, and type of metastases (Appendix 2 for full details).

2.6. Toxicity and disease evaluation

Severe toxicity and serious adverse events were recorded on the end of treatment form but as a registry this was not graded according to the National Cancer Institute Common Toxicity Criteria.

If no signs of tumour progression were present, a formal tumour revaluation was advised at the end of treatment in patients without measurable disease and after 12 weeks of chemotherapy in patients with measurable disease, including those patients with metastases.

2.7. Statistical analyses

Data were collected via a web-based system and analysed at Istituto Oncologico Veneto (Padua, Italy) considering information reported up to 27th May 2015. Continuous variables were summarised with median, minimum and maximum, and categorical variables were reported as counts and percentages. Survival time was calculated from the date of diagnosis to the time of event or last follow-up. Tumour progression, relapse or death due to any causes were considered for event-free survival (EFS). OS was measured from the date of diagnosis to death for any reason. Patients still alive at the end of the study were censored at the date of last observation. The survival probability was computed by means of the Kaplan–Meier method and heterogeneity in survival among strata of selected variables was assessed through the log-rank test. The 3-year EFS and OS (4-year EFS for localized tumours) were reported along with their 95% confidence intervals (CIs). To investigate the impact of the variables gender, age category ($\leq$1 year; >1 year), tumour size ($\leq$5 cm; >5 cm), primary site (favourable: orbit, head and neck non-parameningeal, genitourinary non-bladder–prostate; unfavourable: parameningeal, bladder-prostate, extremities, "other"; according to rhabdomyosarcoma classification, IRS group and initial surgery (performed; not performed) on EFS for localized patients and OS for localized and metastatic patients, survival multivariable analysis were conducted using the Cox proportional hazard regression method [8]. A stepwise variable selection procedure was applied to the covariates with a p-value of at least 0.05 at univariate analysis. Hazard ratios (HRs) with their 95% CI calculated according to the Wald method was reported for significant variables. To check the proportional hazards assumption, a score process (which is a transformed partial sum process of the martingale residuals) was compared with the simulated processes under the null hypothesis that the proportional hazards assumption holds [14]. All data analyses were performed using the SAS statistical package (SAS, release 9.4; SAS Institute Inc, Cary, NC).

3. Results

3.1. Patients

Between December 2005 and June 2014, 110 patients were enrolled on the study but 10 were excluded due to adherence to other protocols (3), immunohistochemistry and molecular data missing (1), histological diagnosis after pathology review was not MRT (2) or immunohistochemistry did not demonstrate loss of nuclear expression of INI-1, and/or molecular testing did not demonstrate deletion of the *SMARCB* gene (4), leaving in total 100 eligible patients. There was an even distribution between the sexes, 49 female and 51 male. The median age at diagnosis was 1.4 years (range 3 d–10.9 years) with 41 patients $\leq$1 year of age. The majority (56 patients) were between 2 and 9 years (39 patients between the ages 1 and 3 years) and only 3 were older than 10 years. Patient staging data and site and size of primary tumour are listed in Table 1. The majority in the series had localised disease (77 patients) and of those 19 (25%) had surgical resection up front. The primary site of the tumour was across multiple

Table 1
Clinical characteristics.

	Localised patients, N = 77	Metastatic patients, N = 23	Total	Total %
Age (years) at diagnosis				
Median (min–max)	1.51 (0.01–10.93)	0.60 (0.01–0.60)	1.38 (0.01–10.93)	
≤1	29	12		41
>1	48	11		59
Gender				
Female	35	14		49
Male	42	9		51
Post-surgical tumour staging (IRS)				
Group I	7	–		7
Group II	12	–		12
Group III	58	–		58
Group IV	–	23		23
Primary tumour Invasiveness (T)				
T0—no detectable	–	1		1
T1—localized to the organ or tissue of origin	34	6		40
T2—extending beyond the tissue or organ of origin	42	14		56
Tx—insufficient information about the primary tumour	1	2		3
Tumour size				
a: ≤5 cm	19	3		22
b: >5 cm	56	19		75
X: not evaluable	2	1		3
Regional lymph node involvement				
N0—No evidence of lymph node involvement	67	10		77
N1—Evidence of regional lymph node involvement	9	11		20
Nx—No information on lymph node involvement	1	2		3
Site of origin of primary tumour				
Orbit	1	–		1
Head neck	12	–		12
Parameningeal	7	–		7
Bladder-prostate	4	–		4
Genitourinary non-Bladder–prostate	11	7		18
Kidney	*10*	*7*		*94*
Uterus	1	–		5
Extremities	8	6		14
Other sites	34	10		44
Abdomen	*2*	–		*2*
Liver	*10*	*5*		*15*
Paraspinal	*13*	*1*		*14*
Pelvis	*1*	–		*1*
Perineum	*1*	–		*1*
Retroperitoneal	–	*2*		*2*
Thorax	*6*	*2*		*8*
Trunk	*1*	–		*1*
Number of metastatic sites[a]				
1	–	9		39
2	–	8		35
3	–	3		13
4	–	3		13

IRS, Intergroup Rhabdomyosarcoma Study; max, maximum; min, minimum.

[a] Percentage computed considering metastatic patients only.

anatomical sites, the commonest site in this series was "other" sites (44 patients) followed by genitourinary non-bladder–prostate (18 cases).

Twenty-three patients had distant metastases. The majority (17 patients) had metastases to the lung: four patients lung alone and 13 with other metastases. Two cases had brain tumour metastases. Thirteen patients had congenital MRT as defined by diagnosis within the first 4 weeks of birth. Five of them (39%) had metastatic disease, with the majority having tumours greater than 5 centimetres (62%). Primary sites were multiple but the largest group was "other"—paraspinal, thorax, retroperitoneal or liver. One case had brain tumour metastases.

3.2. Treatment and toxicity

Forty-three patients completed the protocol treatment in a median period of 8.4 months (minimum 6.5–maximum 13.0) of chemotherapy. Fifty-five patients discontinued chemotherapy due to toxicity (3), early progressive disease (49) between 3 d and 10.9 months or physician's choice (3). One patient did not receive any treatment due to death before starting treatment and for one patient no treatment data are available. There were dose adjustments due to delays in starting the next course of chemotherapy or mucositis in 10 patients. The most frequent reported toxicities included bone marrow suppression, febrile neutropenia, infection, mucositis, anorexia, and electrolyte disturbances. In those who completed all courses of chemotherapy, there were no permanent toxicities, such as renal impairment, and there were no toxic deaths. All those younger than 12 months were able to receive chemotherapy except one patient who died before the start of treatment. They were no more likely to have toxicities than older patients but had doses of chemotherapy adjusted for their age and weight.

Fifty-four patients from the whole cohort did not receive radiotherapy, 39 had progressive disease during first-line treatment prior to the planned radiotherapy, whereas in 15 patients, no radiotherapy was delivered by physicians choice probably due to the very young age of the patient. One patient developed radiation colitis but there were no other radiation-recorded toxicities. For the localised patients, 25 progressed before planned radiotherapy with only 37 of the remaining 52 patients receiving radiotherapy.

Up-front complete surgical resection of the primary tumour was performed in 8 (R0 resection), including 1 metastatic patient, and in 12 patients R1 resection. In 73, only a surgical/trucut biopsy or lymph node exploration was performed at diagnosis (53 localised and 20 metastatic patients). For the remaining seven patients, macroscopic tumour was present after surgical resection of primary tumour (five localised and two metastatic patients). Thirty-nine patients had second surgery, for

26 after 3–4 cycles of chemotherapy, for 8 after 5–8 cycles and for 3 at another time. Additional surgeries were necessary for two patients. This resulted in a 17 with a R0 resection including 1 with a liver transplant, R1 resection in 13, and macroscopic residual tumour in 8. In one case, no tumour was found.

3.3. Outcome data

Median follow-up for alive patients of the complete cohort was 44.6 months (range 11.5–84.6), for localized patients was 49.8 months (range 11.5–84.6), whereas for metastatic patients was 32.1 months (range 14.9–38.8). Sixty-seven patients developed an event (46 in localized and 21 metastatic patients) and subsequently 65 died (45 in localized and 20 metastatic patients). Median time to progression was 5.0 months (minimum 3 d, maximum 31.5 months), for localised patients 7.5 months (1.4–31.5) and for metastatic patients 2.7 months (3 d–14.9 months). In the total cohort, 35 were alive at the time of this analysis.

For the whole cohort, the 3-year EFS was 32.3% (95% CI 23.2–41.6%) with a 3-year OS of 38.4% (95% CI 28.8–47.9%; Fig. 1A and B). For localized disease, the 4-year EFS was 39.3% (95% CI 28.2–50.1%) with a 4-year OS of 40.1% (95% CI 28.4–51.5%; Fig. 1C and D). For metastatic disease, the 2-year EFS was 8.7% (95% CI 1.5–24.2%) with a 2-year OS of 13.0% (95% CI 3.3–29.7%; Fig. 1C and D). For IRS III disease, achieving a complete response (CR) at any time point occurred in 30 patients leading to a statistically significant ($p<0.0001$) survival advantage with a 4-year EFS of 66.3% (95% CI 46.5–80.3%) and 4-year OS of 66.8% (95% CI 44.6–81.7%) compared with no CR in 28 patients with a 4-year EFS of 4.8% (95% CI 0.4–18.9%) and 4-year OS of 4.8% (95% CI 0.4–18.9%).

3.4. Prognostic factors

Table 2 lists the estimated EFS and OS for the patient's clinical characteristics in those with localized tumours. On univariate analysis, patient age only significantly influenced the EFS and OS, with those ≤1 year of age having a significantly worse outcome, with a 4-year EFS of 17.2% (95% CI 6.3–32.7%) and an HR of 2.9 (95% CI 1.6–5.3) and a 4-year OS of 20.1% (95% CI 7.9–36.3%) with an HR of 2.7 (95% CI 1.5–5.0). Table 3 lists the estimated 1-year OS by main characteristics of

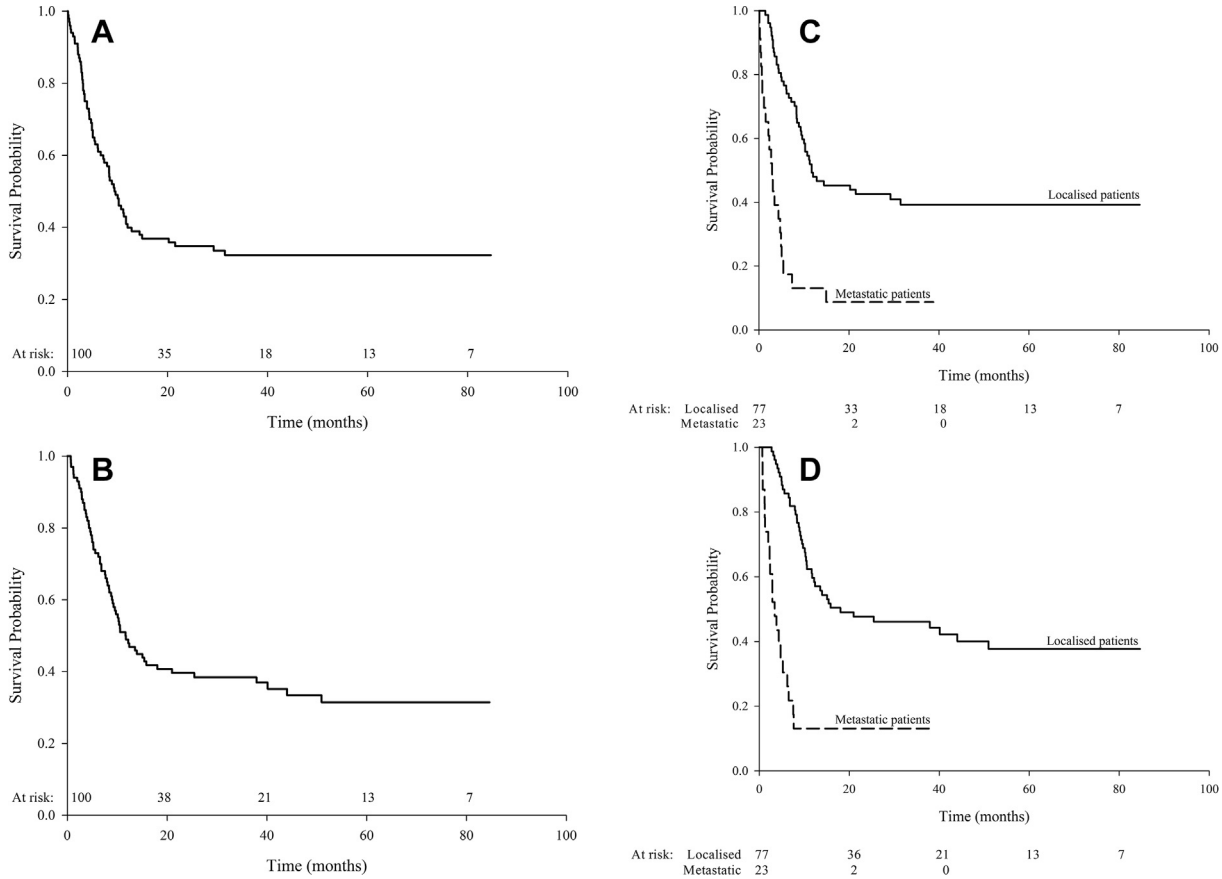

Fig. 1. Survival analysis of whole cohort, localised and metastatic extracranial MRT. (A) Event-free survival and (B) overall survival of 100 patients with extracranial MRT registered on EpSSG NRSTS 2005. (C) Event-free survival and (B) overall survival of localised and metastatic patients separately.

Table 2
Estimated EFS and OS for localised patients (univariate analysis).

Characteristics	N	No. events EFS	1-Year EFS (95% CI)	4-Year EFS (95% CI)	*p*-Value	No. events OS	1-Year OS (95% CI)	4-Year OS (95% CI)	*p*-Value
IRS group					0.2961				0.3234
I	7	2	85.7 (33.4–97.8)	68.6 (21.3–91.2)		2	85.7 (33.4–97.8)	68.6 (21.3–91.2)	
II	12	8	41.7 (15.2–66.5)	33.3 (10.3–58.8)		8	75.0 (40.8–92.2)	41.7 (15.2–66.5)	
III	58	36	44.8 (31.8–57.0)	36.9 (24.4–49.4)		35	53.4 (39.9–65.2)	35.9 (22.7–49.3)	
Age at diagnosis (years)					0.0002				0.0005
≤1 year	29	24	24.1 (10.7–40.5)	17.2 (6.3–32.7)		23	34.5 (18.2–51.4)	20.1 (7.9–36.3)	
>1 year	48	22	62.4 (47.2–74.4)	52.8 (37.5–66.1)		22	75.0 (60.2–85.0)	52.1 (35.7–66.2)	
Gender					0.6600				0.6288
Male	42	23	45.0 (29.6–59.2)	45.0 (29.6–59.2)		23	61.8 (45.4–74.6)	45.6 (29.5–60.4)	
Female	35	23	51.4 (34.0–66.4)	33.3 (18.3–49.1)		22	57.1 (39.3–71.5)	33.4 (17.4–50.3)	
T[a]					0.2193				0.1196
T0–T1	34	17	55.7 (37.6–70.5)	49.3 (31.6–64.8)		16	64.6 (46.1–78.1)	52.4 (32.7–68.9)	
T2	42	28	42.8 (27.8–57.0)	32.6 (19.0–47.0)		28	57.1 (40.9–70.4)	31.9 (18.2–46.5)	
Size[a] (cm)					0.6555				0.6671
≤5	19	10	52.6 (28.7–71.9)	47.4 (24.4–67.3)		10	63.2 (37.9–80.4)	47.4 (24.4–67.3)	
>5	56	34	48.1 (34.5–60.4)	37.4 (24.4–50.3)		33	60.6 (46.6–72.0)	38.0 (24.0–51.9)	
Site					0.2765				0.3525
Favourable	24	12	57.8 (35.7–74.7)	48.9 (27.8–67.0)		12	75.0 (52.6–87.9)	52.3 (30.4–70.2)	
Unfavourable	53	34	43.3 (29.9–56.1)	35.5 (22.8–48.3)		33	52.8 (38.6–65.1)	35.5 (22.3–48.9)	
Initial surgery					0.7451				0.8096
No	49	29	44.9 (30.7–58.1)	40.2 (26.4–53.7)		28	55.1 (40.2–67.7)	39.7 (24.9–54.1)	
Yes	28	17	53.3 (33.5–69.7)	37.2 (19.4–55.1)		17	67.7 (47.0–81.7)	40.0 (21.5–57.9)	

CI, confidence interval; EFS, event-free survival; IRS, Intergroup Rhabdomyosarcoma Study; OS, overall survival.
[a] The sum does not add up to the total because of missing values.

metastatic patients. Patients ≤1 year of age had the worst prognosis, as well as male patients. Multivariable analysis disclosed that all patients ≤1 year were associated with at higher risk of death (HR: 2.6; 95% CI 1.0–6.8; p-value = 0.0094). Risk of death was also related with gender in metastatic patients (HR for males: 2.9, 95% CI 1.0–8.0; p-value = 0.0077)

Table 3
Estimated OS for metastatic patients (univariate analysis).

Characteristic	N	No. events	1-year OS (95% CI)	*p*-Value
Age at diagnosis (years)				0.0094
≤1 year	12	12	0	
>1 year	11	8	27.3 (6.5–53.9)	
Gender				0.0077
Male	9	9	0	
Female	14	11	21.4 (5.2–44.8)	
T[a]				0.3709
T0–T1	7	7	0	
T2	14	11	21.4 (5.2–44.8)	
Size[a]				0.1913
≤5 cm	3	2	33.3 (9.0–77.4)	
>5 cm	19	17	10.5 (1.8–28.4)	
Site				0.6406
Favourable	7	7	0	
Unfavourable	16	13	18.8 (4.6–40.2)	
Initial surgery				0.4330
No	19	17	10.5 (1.8–28.4)	
Yes	4	3	25.0 (8.9–66.5)	

CI, confidence interval; OS, overall survival.
[a] The sum does not add up to the total because of missing values.

4. Discussion

Our results demonstrate that in this first large prospective study of extracranial MRT treated in multiple European countries for what is a very rare soft tissue sarcoma, intensive therapy can be delivered to a very young paediatric population of patients, with possibly an improvement in outcome, be it in comparison with historical series. Furthermore, a substantial proportion of the patients in this EpSSG protocol had an extra-renal tumour site, which confers a poorer prognosis [1]. The outcome remains poor for the majority of patients in this series, in particular patients with metastatic disease and those who progressed, who universally had a fatal outcome.

In the NWTS series of renal MRT, over a much longer historical period between 1969 and 2002, OS at 4-year was 23.2% [3]. This compares to, perhaps, our superior results with an OS of 38.4%, and perhaps, it is significant in terms of a better outcome, as the NWTS series only contained patients with a renal primary, thought to have a better outcome, maybe in part because a larger proportion can have up-front resection of the primary tumour. In our series, it is noteworthy that only 24% had up-front surgery with no survival advantage, and with surgery following chemotherapy 73% were in CR. CR, by either surgery or chemotherapy in IRS III patients, had a survival advantage but also reflects those patients who had not progressed before delayed local control and, therefore, must be read with caution. The role of a CR maybe important for

long-term survival as suggested in previous small series [11]. The small numbers with a concomitant CNS primary compared to the NWTS series reflect selection of these patients into CNS protocols rather than our study [3]. The small numbers also makes it hard to comment on therapy, but at present most will receive similar intensive chemotherapy, surgical resection if possible plus or minus radiation.

We showed that age continues to be an important prognostic factor and remains the only factor in multivariable analysis for OS in localised patients and univariate analysis for metastatic patients. The importance of age, in particular the negative effect of younger age on outcome, confirms the findings in the NWTS series [3], the SEER database series [4], and the UK population-based registry [1]. Uniquely, we analysed the congenital cases separately (13 cases) with 12 events (all died) and a median time to event of 3.1 months (3–11.7 d). It might be expected that these cases had a germline mutation of the *SMARCB1* gene, thought to confer a poorer prognosis, but our data are incomplete [12]. Their outcome may also question the role of intensive therapy in congenital cases or, indeed, in the very young cohort. For parents, however, offering palliative therapy as the first line of treatment may not be acceptable.

Progression on treatment remains an important finding, 49.5% progressed on treatment, which was an important factor for those subjects not receiving the recommended protocol radiotherapy. Of course, age of the patient may also be a further factor for no radiotherapy as in the 15 patients with physicians choice for no radiotherapy, 14 were younger than 2 years.

The role of radiotherapy as an important factor affecting outcome could not be shown in our series, confounded by the number who progressed prior to delivering radiotherapy and the reluctance to give radiotherapy to very young children especially in infants or with a planned delay. This echoes the findings of the NWTS series, as the possible benefit of radiotherapy again was difficult to define, and also confounded by the patient's age [3]. Radiotherapy tended to be given to those with a higher clinical stage and in an older age group, who received a higher dose. This is in contrast, however, to the SEER database series [4]. In particular where the use of radiotherapy remained a significant predictor of survival ($p = 0.0006$). Radiotherapy was only used in 35% of patients in total, but there was no significant difference in its use at the different primary tumour sites ($p = 0.90$). Less was used, however, in those younger than 3 years.

For localised disease, stage was not an important predictor of outcome but a statistically significant difference in EFS and OS is evident comparing localised with metastatic patients (p-value for log-rank test <0.0001 in EFS and OS). In both the NWTS series and the SEER database series, stage also determined outcome, with a 41.8% 4-year OS for stage I to II tumours compared with 15.9% in those with stage III, IV, or V disease in NWTS, and in for the SEER database series in a multivariable model applied only to children and adolescents with extracranial MRT, tumour stage remains a significant predictor of survival ($p = 0.00014$) [3,4].

Like any discussion comparing historical series, the staging systems used, the patient selection and the numbers at each anatomical site are not directly comparable and, hence, cannot replace a randomised study. The lack of prospective historical series in MRT at all sites hampers this further. Extracranial MRT continue to be aggressive tumours with poor survival. The young age at presentation often limits the ability to deliver multimodal therapy, in particular radiotherapy, which seems to be important. Further research needs to allow better understanding of MRT biology and the role of the *SMARCB1* gene in MRT development. The later information could also determine better and more targets for therapy. A recent eloquent study in the molecular subgroups of primary brain atypical teratoid rhabdoid tumours, biologically the same tumour, allowed further stratification of these tumours for future biologically based trials [10]. Our results may allow us to use this protocol as a standard chemotherapy backbone in order to add small molecule inhibitors against what we currently know are targets. Recent data on EHZ2 inhibitors is promising as an epigenetic regulator and should be in phase I studies shortly in children [13]. We may need to take a leap of faith based on cell line data and pre-clinical mouse models to put these agents into phase III clinical trials while not having data from phase II trials in MRT, as it is so rare, but at least toxicity data from phase I studies in paediatric tumours. We will not improve the outcome with this protocol which is already at maximal tolerance but we may alter how we deliver conventional chemotherapy as successfully demonstrated in the Ewings sarcoma study of interval compressed chemotherapy—AEWS0031 [9], with new targeted agents, to be given in combination, in a multiple arm randomised study using an innovative statistical plan for rare cancers.

Conflict of interest statement

None declared.

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Appendix 1. Chemotherapy schedule and drug doses for rhabdoid tumours registered on the EpSSG NRSTS 2005 study

Chemotherapy schedule

Week number

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
V	V	V							V	V	V	V	V	V							V	V	V				V	V	V
D			Cy*			Cy*			D			D			Cy*			Cy*			D			Cy*			D		
Cy			C			C			Cy			Cy			C			C			Cy			C			Cy		
			E			E									E			E						E					

V	Vincristine	0.025 mg/kg/d i.v. × 1 as bolus for infants <12 months 0.05 mg/kg/d i.v. × 1 as bolus for children 12 months to 3 years 1.5 mg/m^2/d × 1 as bolus for children ≥3-year old
D	Doxorubicin	1.25 mg/kg/d i.v. × 2 d over 15 min for infants <12 months 37.5 mg/m^2/d i.v. × 2 d over 15 min for children ≥12 months
Cy	Cyclophosphamide	40 mg/kg/d i.v. × 1 d over 1 h for infants <12 months 1200 mg/m^2/d i.v. × 1 d over 1 h for children ≥12 months
Cy*	Cyclophosphamide	14.7 mg/kg/d i.v. over 1 h × 5 d for infants <12 months 440 mg/m^2/d i.v. over 1 h × 5 d for children ≥12 months
C	Carboplatin	See nomogram in protocol
E	Etoposide	3.3 mg/kg/d i.v. over 1 h × 5 d for infants <12 months 100 mg/m^2/d i.v. over 1 h × 5 d for children ≥12 months

Administration schedule for cycles VDCy weeks 1, 10, 13, 22 and 28

Drug	Route	Dose	Week (s)	Day(s)
Vincristine	i.v. over 1 min	0.025 mg/kg/d for infants <12 months 0.05 mg/kg/d for children 12 mo. to 3 years 1.5 mg/m^2/d for children ≥3-year old	1, 2, 3, 10, 11, 12, 13, 14, 15, 22, 23, 24, 28, 29, 30	1
Doxorubicin	i.v. over 15 min	1.25 mg/kg/d for infants <12 months 37.5 mg/m^2/d for children ≥12 months Consideration for the use of a cardioprotective agent. Individual groups may prefer to infuse over 1 h.	1, 10, 13, 22, 28	1–2
Cyclophosphamide with MESNA hydration[a]	i.v. over 1 h	40 mg/kg/d for infants <12 months 1200 mg/m^2/d for children ≥12 months	1, 10, 13, 22, 28	1

[a] MESNA and hydration guidelines: MESNA 1440/m^2/dose (48 mg/kg/dose for infants <12 months old) should be added to the hydration (2000 ml/m^2/16 h) of 0.45% saline/2.5% dextrose and run for 3 h pre- and with cyclophosphamide and at least 12 h post-cyclophosphamide—total 16 h. Urine output at least 3 ml/kg/h.

Administration schedule for cycles Cy*CE weeks 4, 7, 16, 19, and 25

Drug	Route	Dose		Week	Day(s)
Cyclophosphamide with MESNA hydration	i.v. over 1 h	14.7 mg/kg/d for infants <12 months 440 mg/m^2/d for children ≥12 months		4, 7, 16, 19, 25	1–5
Carboplatin	i.v. over 1 h	GFR	Dose	4, 7, 16, 19, 25	1
		>150 ml/min/1.73 m^2	560 mg/m^2 (18 mg/kg for infants)		
		100–150 ml/min/1.73 m^2	500 mg/m^2 (16.6 mg/kg for infants)		
		75–99 ml/min/1.73 m^2	370 mg/m^2 (12.3 mg/kg for infants)		
		50–74 ml/min/1.73 m^2	290 mg/m^2 (9.7 mg/kg for infants)		
		≤49 ml/min/1.73 m^2	Discuss with study coordinators		
Etoposide	i.v. over 1 h	3.3 mg/kg/d for infants <12 months 100 mg/m^2/d for children ≥12 months		4, 7, 16, 19, 25	1–5

Hydration: prehydrate with 0.45% saline/2.5% dextrose at 125 ml/m^2/h for 2 h. Then continue at 125 ml/m^2/h for 2 h following completion chemotherapy—total fluids 500 ml/ m^2 with 530 mg/m^2 of MESNA added.
i.v., intravenous; MESNA, 2-mercaptoethane sulfonate sodium.

Appendix 2. Radiotherapy guidance for the rhabdoid tumours registered on the EpSSG NRSTS 2005

Renal rhabdoid tumours

Indications for post-operative flank radiotherapy

- Stage I–III renal rhabdoid tumour (19.8 Gy in 11 fractions of 1.8 Gy over 15 d for patients ≥ 12 months; 10.5 Gy in 7 fractions of 1.5 Gy over 9 d for patients < 12 months)

Indications for whole-abdominal and pelvic radiotherapy

- Stage III with cytology positive ascites
- Pre-operative intraperitoneal rupture
- Diffuse operative spill and peritoneal seeding (19.5 Gy in 13 fractions of 1.5 Gy over 17 d for patients ≥ 12 months; 10.5 Gy in 7 fractions of 1.5 Gy over 9 d in the case of infants)

Indications for pulmonary radiotherapy

- Lung metastases (15 Gy with lung correction in 10 fractions of 1.5 Gy over 12–14 d for patients ≥ 12 months; 10.5 Gy in 7 fractions of 1.5 Gy over 9 d for patients < 12 months)

Indications for liver radiotherapy

- Liver metastases (19.8 Gy in 11 fractions of 1.8 Gy for patients ≥ 12 months; 15 Gy in 10 fractions of 1.5 Gy for patients < 12 months.)

Indications for whole-brain radiotherapy

- Brain metastases (21.6 Gy in 12 fractions of 1.8 Gy) + boost of 10.6 Gy

Indications for bone metastases radiotherapy

- None metastases (25.2 Gy in 14 fractions of 1.8 Gy)

Timing of radiation therapy

All radiation therapy should begin as soon as it is logistically possible concurrent with the initiation of chemotherapy after surgery which is either up front or after 12 weeks of chemotherapy.

Equipment

All patients will be treated with megavoltage equipment (4–20 MV linear accelerator. The use of colbalt-60 equipment is not acceptable for radical therapy.)

Treatment planning

All patients should have a planning CT scan to enable three-dimensional conformal planning, generation of dose volume histograms for organs at risk, and lung correction where necessary. The dose is prescribed according to international commission on radiation units and measurements (ICRU) 50.

Fractionation

Treatment is given with conventional fractionation, treating all fields each day, with one treatment daily, 5 d a week. The fraction size should be 1.8 Gy except with large fields (whole-abdominal and pelvic radiotherapy, and whole-lung irradiation) and in infants. Once treatment is started, there will be no interruptions in treatment unless absolutely necessary. It is not necessary to suspend treatment because of uncomplicated myelosuppression, supportive care should be given for neutropenia and thrombocytopaenia according to local protocols. Haemoglobin levels should be maintained at 12 g/dl or above during the time of radiotherapy.

►►► *Compensation for treatment breaks*

Standard fractionation is one treatment per day, 5 d each week. If a treatment interruption is unavoidable, this should be compensated for. Ideally, two fractions per day with a minimum interfraction interval of 6 h should be given to enable treatment to be completed within the same overall time as was originally intended. If this is not possible, for example in the case of a child requiring general anaesthesia, one or two additional fractions should be given according to the Children's Oncology Group (COG) guidelines below.

Or as per COG protocol

The total number of fractions or total radiotherapy dose to be delivered according to the duration of interruptions is indicated below:

Patients prescribed 10.8 Gy

Timing	Fx size	# Fx	Total dose (Gy)
Normal and/or up to 3-d split	1.8	6	10.8
4- to 7-d split	1.8	7	12.6
>7-d split	1.8	8	14.4

Patients prescribed 19.8 Gy

Timing	Fx size	# Fx	Total dose (Gy)
Normal and/or up to 3-d split	1.8	11	19.8
4- to 7-d split	1.8	12	21.6
>7-d split	1.8	13	23.4

Target volume definition for primary tumour

- The target volume is chosen according to the initial tumour volume (gross tumour volume [GTV]). The pre-therapeutic CT is usually the optimal imaging study.
- The clinical target volume (CTV) is defined as the GTV + 1 cm extended medially (and superiorly and inferiorly as appropriate) to encompass vertebral bodies in their entirety.
- The planning target volume (PTV) is defined as the CTV + 1 cm unless departmental quality control data indicate that a different margin is appropriate.

Flank irradiation

The GTV is determined by the pre-operative CT scan and it is defined as the outline of the kidney with the

associated tumour. The PTV should not extend more than 2 cm beyond the defined GTV, except where necessary to allow the superior and inferior field borders to lie within an intervertebral space, and the medial border to fully encompass the entire vertebral width without significantly overlapping the contralateral kidney. In patients where the tumour prior to resection bulged into the contra lateral flank without tumour invasion into the contra lateral kidney, it is not necessary for the CTV to encompass the medial extent of the GTV, and so the PTV can lie so that the full vertebral width is covered without overlap of the contralateral kidney. In most patients, the superior border of the radiation therapy field will be well below the diaphragmatic dome. The radiation therapy field should not be extended to the dome of the diaphragm unless there is tumour extension to that height. When there are positive lymph nodes that have been surgically removed, the entire length of the para-aortic chain of lymph nodes should be included in the radiotherapy field. An anteroposterior parallel-opposed (AP-PA) technique is recommended for flank irradiation. The borders of the radiation fields should be placed so that the PTV is encompassed by the 95% isodose. The flank irradiation dose is 19.5 Gy in 13 fractions of 1.5 Gy over 17 d for those 12 months or older, and 10.5 Gy in 7 fractions of 1.5 Gy over 9 d in the case of infants. Dose volume histograms should be performed for liver and the remaining kidney to ensure that the doses to these organs at risk are kept within tolerance levels. At least two thirds of the remaining kidney should not receive a dose greater than 14.4 Gy, and at least half the liver should not receive a dose greater than 19.8 Gy.

Whole-abdominal and pelvic irradiation

For whole-abdominal and pelvic radiotherapy, the CTV will be the entire peritoneal cavity that extends from the dome of the diaphragm superiorly to the pelvic diaphragm inferiorly and laterally from the right to the left lateral abdominal wall. The superior border of the whole-abdominal and pelvic field will be placed approximately 1 cm above the dome of the diaphragm. The inferior border of the field will be placed at the bottom of the obturator foramen. The lateral borders of the field will be placed approximately 1 cm beyond the lateral abdominal wall. The femoral heads should be shielded during radiotherapy. An AP-PA is recommended for whole-abdominal and pelvic irradiation. The dose/fractionation schedule for whole-abdominal and pelvic radiotherapy is 19.5 Gy in 13 fractions of 1.5 Gy over 17 d for those 12 months or older. For these patients, the remaining kidney should be shielded to limit the dose to 14.4 Gy. In the case of infants, the whole-abdominal and pelvic dose is 10.5 Gy in 7 fractions of 1.5 Gy over 9 d. This treatment should be CT planned to allow dose volume histograms to be generated for organs at risk. This is especially important if a second phase of treatment to boost the dose to macroscopic residual disease is being contemplated (Section 9.1.8).

Boost for gross residual disease

Patients with gross residual disease after surgery may receive a second phase of treatment after flank or whole-abdominal and pelvic radiotherapy. This requires individualised consideration. Depending on factors such as the volume which would require treatment, and the age of the patient, a lower dose may be deemed safer, or the boost may be omitted. The GTV will be defined on the post-operative planning CT scan used for planning the first phase of treatment. The CTV will usually be the same as the GTV, but may be extended to ensure uniform irradiation of vertebral bodies. The PTV will be the CTV + 1 cm unless departmental quality control data indicate that a different margin is appropriate. The organs at risk will already have been delineated on the planning CT scan. Fields will be shaped with multileaf collimator (MLC) or customised blocks to conform to the PTV. The most appropriate field arrangement will be selected by the clinician taking into account the composite dose volume histograms for phase I and phase II combined, with respect to coverage of the PTV and the dose constraints to organs at risk as stated in Section 9.1.6. The dose will usually be 10.8 Gy in six fractions of 1.8 Gy over 8 d, but 10.5 Gy in seven fractions over 9 d may be more appropriate in infants or if the volume is large.

Whole-lung irradiation

Both lungs are irradiated regardless of the number and location of the metastases. Treatment should be CT planned with patient lying supine with the arms to the side, slightly away from the body. The CTV includes the entire lungs, mediastinum and the pleural recesses. The CTV to PTV margin should take account of respiratory movement and is likely to be about 1 cm superiorly and laterally and 2 cm inferiorly. AP-PA and posterior parallel-opposed field will be used such that the PTV is encompassed with the 95% isodose. CT planning will take into account and correct the increased transmission through lung tissue. The inferior border of the field should lie in an intervertebral space, often below L1. The shoulder joints should be protected by MLC or cerrobend shielding. The whole-lung irradiation (WLI) dose/fractionation schedule for those aged 12 months or over is 15 Gy with lung correction in 10 fractions of 1.5 Gy over 12–14 d. For infants, it is 10.5 Gy in seven fractions of 1.5 Gy over 9 d. If patients require both whole-lung and infra-diaphragmatic irradiation, then both fields should be treated simultaneously whenever possible. As the volumes for WLI often abut or overlap with the volumes for flank or whole-abdominal and pelvic radiotherapy, the contiguous areas should be treated in

the first instance as a single volume with a single pair of appropriately shaped AP-PA and posterior parallel-opposed fields. For such a large volume, a fraction size of 1.5 Gy will be used. The fields will be reduced in size (off the lungs) after 10 fractions (15 Gy) to cover only the infra diaphragmatic volume. If the WLI volume and the flank volume appear well separated, they may be treated simultaneously as two separate areas, but great care must be taken when planning to ensure an adequate gap so that there is no overlap. Similarly, if WLI and infra-diaphragmatic radiotherapy are given at different times, care must be taken to ensure that there is no overlap.

Localized foci of lung disease persisting 2 weeks after the delivery of WLI may either be excised or given an additional 7.5 Gy in five fractions. The volume of the lungs included in this boost irradiation field should be <30% in order to limit the acute and long-term pulmonary complications that could result from higher doses of irradiation.

Liver irradiation

The entire liver is included in the irradiation portal only if the liver is diffusely involved (19.8 Gy in 11 fractions of 1.8 Gy.) In infants the dose/fractionation schedule should be 15 Gy in 10 fractions of 1.5 Gy. If the entire liver volume is not involved, then only the metastases with a margin of 2 cm is irradiated. Additional boost irradiation doses of 5.4 to 10.8 Gy may be administered to limited volumes (<75% of the entire liver) at the discretion of the clinical oncologist. While irradiating the liver, the dose to the upper pole of the remaining kidney should be monitored. A posterior kidney block may be inserted in order to limit the remaining kidney to ≤14.4 Gy. An AP-PA technique is recommended for liver irradiation.

Brain irradiation

In patients with brain metastases, the whole brain is included in the irradiation field to a dose of 21.6 Gy in 12 fractions of 1.8 Gy. A boost of at least 10.8 Gy is required to site of metastases. In patients with ≤3 circumscribed lesions especially in patients younger than 3 years, a limited volume (tumour or tumour bed only with 0–1 cm margin) boost dose of 10.8 Gy in 6 fractions using intensity-modulated radiation therapy (IMRT) or sterotactic radiotherapy may be administered after whole-brain irradiation to 21.6 Gy.

A lateral parallel-opposed technique (right and left lateral) is recommended for whole-brain irradiation.

Bone irradiation

In patients with bone metastases, the GTV is the lesion as shown on appropriate imaging, which may include skeletal scintiography, plain radiographs MRI and CT. The clinical target volume will usually include a margin of apparently healthy bone up to 2 cm. A narrower margin may be appropriate where the metastasis is close to the edge of the bone. Irradiation of the epiphyses should be avoided where possible to diminish late effects. An appropriate margin should be added for the PTV, taking into account the technique of immobilisation used. The entire bone need not be irradiated. An AP-PA technique is usually recommended for bone irradiation, depending on the anatomical site. The bone irradiation dose is 25.2 Gy in 14 fractions of 1.8 Gy, but may be modified if appropriate.

Lymph node irradiation

Lymph nodes with metastatic tumour that have not been surgically removed should receive radiation therapy. Groups of lymph nodes which were involved at presentation should be irradiated in their entirety. The GTV will be the nodal area including any residual mass after chemotherapy as defined on the planning CT scan. The CTV will usually be a 1 cm margin around the GTV. The margin for PTV definition will depend on immobilisation and individual departmental data. If vertebrae are to be irradiated, the whole vertebral body shall be included in the fields. For mediastinal and abdominal nodes, a parallel-opposed field arrangement usually gives best coverage of the PTV. Where possible, nodal areas will be treated in continuity with the primary tumour site or other metastatic sites requiring irradiation. The dose will usually be 19.8 Gy in 11 fractions of 1.8 Gy.

Target dose

The daily dose to ICRU prescription points shall be 1.8 Gy, except in younger children (e.g. <3 years) or when large volumes (e.g. whole lung or whole abdomen and pelvis) are to be treated.

Extrarenal non-CNS rhabdoid tumour

All patients should have a consultation by a radiation oncologist at the time of study entry so that the radiation oncologist can assist in providing appropriate staging/grouping of the patient and review the adequacy of the initial diagnostic imaging studies for subsequent local control treatment with RT.

Extrarenal non-CNS rhabdoid tumours	
Gross total resection with no residual disease (microscopic negative margins) (group I)	36 Gy in 20 fractions
Gross total resection with microscopic residual disease (microscopic positive margins) (group II)	45 Gy in 25 fractions
Biopsy only or gross residual disease (group III)	50.4 Gy in 28 fractions

These total doses and fractionation schedules may need to be modified taking into account factors including the age of the child, the volume requiring irradiation, critical normal structures and co-morbidity.

Equipment

Treatment will usually be with x-ray photons of 4–20 MV from a linear accelerator. The use of cobalt teletherapy is not acceptable.

In some circumstances, the use of electrons may result in a more favourable dose distribution.

Similarly, interstitial or intacavitary brachytherapy may be preferable in certain circumstances, such as with tumours at gynaecological, extremity and some non-parameningeal head and neck primary sites. Brachytherapy should not be used without careful discussion and is only appropriate in specialised treatment centres.

Proton therapy is permitted in this study in specialised treatment centres.

Protocol target volumes

Three-dimensional treatment planning is strongly encouraged for patients treated on this study.

All treatment planning, regardless of whether it is standard or three-dimensional conformal/IMRT, will be based upon the following target definitions. Treatment will be prescribed to the PTV, which will be derived from the GTV and CTV as follows:

GTV

The GTV is defined as the pre-treatment visible and/or palpable disease defined by physical examination, operative surgical findings, computer tomography, or magnetic resonance imaging. The T_1 MR image with contrast is usually optimal imaging study. In special circumstances, changes can be made in this definition based upon the post-operative geometry of the target volume. In patients who have undergone primary surgical tumour resection, the entire surgical scar should be included in the GTV. However, in general, the GTV does not change based on any surgical resection or chemotherapy response.

CTV

For all Clinical Groups, the CTV is defined as the GTV + 1.5 cm (but not extending outside of the patient). For some sites, the definition of the CTV is modified to account for specific anatomic barriers to tumour spread. The CTV will always include the entire draining lymph nodes chain if the regional nodes are clinically or pathologically involved with tumour. Patients with gross residual disease and primary sites in the head and neck and vulva/uterus who do not undergo second look operations may have second CTV and PTV defined for a cone down boost. The patients will receive a total dose of 50.4 Gy given to the PTV.

PTV

For all Clinical Groups, the PTV is defined as the CTV plus an institution specific margin to account for day-to-day setup variation related to the ability to immobilise the patient and physiological motion of the CTV.

Planning organ-at-risk volume

Planning organ-at-risk volumes (PRV) will be defined for each organ at risk defined in Section 14, Radiotherapy Guidelines, and for any other organ that the treating clinical oncologist wishes to limit to a specific dose. The PRV is defined as the volume of the organ at risk plus a margin to account for that organ's positional uncertainty.

Special modifications of GTV and CTV for certain sites

➢*Orbit:*. For orbit primaries, the CTV will not extend outside the bony orbit, providing there is no bone erosion of the orbit.

➢*Thorax:*. Tumours which have displaced a significant amount of lung parenchyma which has subsequently returned to normal anatomic position following surgical debulking will have the GTV defined as the pre-operative tumour volume excluding the intra-thoracic tumour which was debulked. However, all areas of pre-operative involvement of the pleura will be included in the GTV.

➢*Bladder/prostate, perineum, pelvis, biliary tree and abdomen:*. Tumours which have displaced a significant amount of bowel which has subsequently returned to normal anatomic position following surgical debulking will have the GTV defined as the pre-operative tumour volume excluding the intra-abdominal or intra-pelvic tumour which was debulked. However, all areas of pre-operative involvement of the peritoneum or mesentery, and the site of origin, will be included in the GTV.

Timing of radiotherapy:. All patients who require radiation therapy shall begin treatment concurrent with the initiation of chemotherapy after surgery. If surgery is performed up front, radiation therapy should begin as close to the beginning of chemotherapy as possible. If surgery is delayed, radiation therapy should begin after recovery from surgery when chemotherapy is reinitiated. Chemotherapy will be given concurrent with radiotherapy. The regimen is designed so that doxorubicin is avoided during the six weeks following irradiation.

Prescribed dose and fractionation

The total radiotherapy dose for the various clinical groups are indicated in the table below:

Gross total resection with no residual disease (negative margins) (Group I)	36 Gy in 20 fractions
Gross total resection with microscopic residual disease (positive margins) (Group II)	45 Gy in 25 fractions
Biopsy only or gross residual disease (Group III)	50.4 Gy in 28 fractions

All radiation should be given at 1.8 Gy per fraction with one fraction given per day. Five fractions should be given per week.

Interruptions

Patients requiring an interruption in radiotherapy (i.e. for low counts, infection, toxicity) will receive a modification in the schedule as shown in the tables below

Patients prescribed 36 Gy (Gp I)

Timing	Fx size (Gy)	# Fx	Total Dose (Gy)	Total time
Normal and/or up to 2-week split	1.8	20	36	4–6 Weeks
2- to 3-week split	1.8	21	37.8	6–7 Weeks
>3-week split	1.8	22	39.6	>7 Weeks

Patients prescribed 45.00 Gy (Gp II)

Timing	Fx size (Gy)	# Fx	Total dose (Gy)	Total time
Normal and/or up to 2-week split	1.8	25	45	5–7 Weeks
2- to 3-week split	1.8	26	46.8	7–8.4 Weeks
>3-week split	1.8	27	48.6	>8.4 Weeks

Patients prescribed 50.40 Gy (Gp III)

Timing	Fx size (Gy)	# Fx	Total dose (Gy)	Total time
Normal and/or up to 2-week split	1.8	28	50.4	5.4–7.3 Weeks
2- to 3-week split	1.8	29	52.2	7.4–8.4 Weeks
>3-week split	1.8	30	54.0	>8.4 Weeks

Normal tissue sparing

It is important to protect normal vital structures whenever possible. Such shielding must be weighed against the possibility of under treatment of known tumour-bearing tissue.

The recommended upper dose limits for different organs are shown in the table below. These limits are the same as, or less than, those used in the previous IRS studies and have not been associated with excessive toxicity when used with chemotherapy.

Normal tissue tolerance

Organ	Dose limit (Gy)
Optic nerve and chiasm	50
Lacrimal gland	41.4
Small bowel	45.0
Spinal cord	45.0
Lung (when $>1/3$ but $<1/2$ of total lung volume is in the PTV)	18.0
Lung (when $>1/2$ of total lung volume is in the PTV)	15.0
Whole kidney	19.8
Whole liver[a]	23.4

[a] Tolerance for partial liver radiation: when two third of the liver volume is included in the initial radiation port and more than one third of the liver requires a boost beyond the maximum whole liver dose (23.4), the total dose to the boost volume may be limited to a maximum of 30 Gy. The boost volume should not exceed two third of the total liver volume.

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Targeted treatment of severe vascular malformations harboring *PIK3CA* and *TEK* mutations with alpelisib is highly effective with limited toxicity

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This was a prospective cohort study of eighteen patients with large and debilitating vascular malformations with one or more major systemic complications. In all patients, we discovered activating alterations in either *TEK* or *PIK3CA*. Based on these findings, targeted treatment using the PI3K inhibitor alpelisib was started with regular check-ups, therapy duration varied from 6 to 31 months. In all patients, marked improvement in quality of life was observed. We observed radiological improvement in fourteen patients (two of them being on combination with either propranolol or sirolimus), stable disease in 2 patients. For 2 patients, an MRI scan was not available as they were shortly on treatment, however, a clinically visible response in size reduction or structure regression, together with pain relief was observed. In patients with elevated D-dimer levels before alpelisib administration, a major improvement was noted, suggesting its biomarker role. We observed overall very good tolerance of the treatment, documenting a single patient with grade 3 hyperglycemia. Patients with size reduction were offered local therapies wherever possible. Our report presents a promising approach for the treatment of VMs harboring different targetable *TEK* and *PIK3CA* gene mutations with a low toxicity profile and high efficacy.

Abbreviations

BSA	Body surface area
CTCAE v5.0	Common terminology criteria for adverse events version 5.0
NGS	Next-generation sequencing
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PROS	PIK3CA-related overgrowth syndrome
QoL	Quality of life
TEK	Tyrosine kinase, endothelial
VA	Vascular anomalies
VM(s)	Vascular malformation(s)

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VeM	Venous malformation
WES	Whole-exome sequencing

Vascular anomalies (VA) are a large and diverse group of diseases characterized by abnormal growth and development of blood or lymphatic vessels. They are associated with diverse symptomatology and often disabling conditions and remain both diagnostic and therapeutic challenges for medical professionals all over the world[1,2]. According to the ISSVA 2018 classification (Classification of Vascular Anomalies ©2018 International Society for the Study of Vascular Anomalies Available at "issva.org/classification" Accessed 01-JUN-2023), they comprise two major categories, vascular tumors and vascular malformations, which can be further divided into several subgroups. Vascular malformations (VMs) can be categorized into simple, combined, vascular malformations of major named vessels and vascular malformations associated with other anomalies.

The overall incidence of congenital VMs in the general population is 1.5%, and approximately two-thirds of cases are of venous predominance[3]. They can present as localized or diffuse lesions, and the symptomatology depends on the localization, extension, and involved anatomical structures. The appearance and symptoms are not static and can often progress during growth spurts and puberty[1,2]. Because of this varied symptomatology, multidisciplinary cooperation is of vital importance. For extensive lesions with vital organ and body part involvement, surgical procedures are very complicated because of their size and/or location. In these patients, other therapeutic options should be considered.

Mutations in genes that are involved in two significant intracellular signaling pathways, RAS/MAPK and PI3K/AKT, have been implicated in the pathophysiology of the majority of vascular malformations[4–6]. This opens the possibility that drugs/inhibitors currently being used in cancer patients may be used to treat patients with VMs[7].

In this prospective observation report, we focused on the effect of the PI3K inhibitor alpelisib, which was recently approved by the FDA and EMA for the treatment of *PIK3CA*-mutated, hormone receptor-negative advanced breast cancer, showing very promising results[8]. Several authors have already demonstrated its effect in patients with *PIK3CA*-related overgrowth syndromes or *PIK3CA*-altered lymphatic malformations, and as a result of the EPIK-P1 clinical study[9], it has recently been approved by the FDA for adult and pediatric patients with severe manifestations of the *PIK3CA*-related overgrowth spectrum[10–13]. In addition to patients with activating *PIK3CA* mutations, alpelisib treatment might also be beneficial for patients with activating *TEK* alterations, as the PI3K/AKT pathway is considered to be a central part of signaling through the TIE2 receptor encoded by this gene[14]. This was recently demonstrated in work published by Remy et al., in which the authors described the efficacy and pharmacokinetics of alpelisib in 3 patients with VMs harboring *TEK* mutation[15].

Results

In each patient, activating mutations in either *TEK* or *PIK3CA* were found. In 12/18 patients, *TEK* exon 17 mutation was found, with *TEK* p.L914F being the most prevalent (9/12). In one of these patients, we found a *TEK* mutation of germline origin (p.Y897C). One patient harbored a truncating mutation in exon 23 of the *TEK* gene. Five patients harbored *PIK3CA* hotspot mutations, all of which have already been observed in patients with VM. In one patient, in addition to the somatic *TEK* mutation found in VA biopsy, a germline *PTPN11* mutation causing Noonan syndrome was detected. The identified variants and the testing methods for each patient are listed in Table 1.

The total duration of alpelisib treatment varied from 6 to 31 months as of May 2023.

In all patients, marked improvement in the quality of life (QoL) was observed. In 12 patients, radiological improvement was documented; in 2 patients, the anomaly size remained stable; in 2 patients, radiological improvement was documented after combining alpelisib with either sirolimus (No. 6) or propranolol (No. 9). For 2 patients (Nos. 16 and 17), an MRI scan was not available due to short period of alpelisib administration. However, rapid clinical response with subjective improvement in QoL was documented. Effect of the treatment for every single patient is summarized in Table 2, percentage of VM size reduction in pre- and posttherapy MRI scans was radiologically measured either as volumatic, or planar change. For this purpose, we excluded two patients on combined treatment and two patients without MRI at time of analysis.

When comparing patients with *TEK* (9 patients) and *PIK3CA* (5 patients) mutation, better radiological response was observed in *PIK3CA* mutation group. For *TEK* mutation group the average volume reduction was 21% and median value was 19%. For *PIK3CA* mutation group the average volume reduction was 53% and median value was 45%. Moreover, both patients with stable disease harbored *TEK* mutation.

In patients with elevated D-dimer levels before alpelisib administration, a major improvement was noted. The D-dimer levels continually decreased, as shown in Fig. 1.

Notable individual cases are further described in more detail below.

We observed overall very good tolerance of the treatment. According to the CTCAE v5.0[16], we noted grade 3 toxicity of hyperglycemia requiring insulin treatment in one patient and grade 2 toxicity in 4 patients. Those specifically included mucositis in two patients, hyperglycemia requiring oral hypoglycemic medication and recurrent abdominal pain in one. The rest of the reported side effects were only grade 1, most commonly temporary abdominal discomfort, headaches, nausea, hyperglycemia, or liver enzyme elevation. Apart from the patient with hyperglycemia on insulin treatment, none of the patients required admission for treatment of adverse events, and all were managed in an outpatient setting.

Index cases presentation. One of the first patients (No. 2) who was administered alpelisib treatment was an 18-year-old woman with severe and debilitating venous malformation of the right lower limb, pelvis, and genitalia. Prior to the start of alpelisib in November 2020, she underwent several surgical procedures over the

Patient	Gender	Age (y)	Gene alteration	Method	Class of VM according to ISSVA classification and clinical manifestation
1	F	9	*TEK* c.2740C > T/p.L914F	WES	VeM of left lower limb, buttocks, and pelvis, intraosseous infiltration, movement limitations, pain
2	F	19	*TEK* c.2740C > T/p.L914F	WES	VeM of right lower limb, buttocks, pelvis, and genitals, chronic DIC, bleeding, immobility, pain
3	F	18	*TEK* c.2740C > T/p.L914F, *PTPN11* c.598A > T/p.N200Y (germline)	WES	VeM of the left scapula and upper limb, chronic DIC, movement limitation, pain
4	F	11	*PIK3CA* c.1258 T > C/p.C420R	WES	VeM of the left chest and abdominal wall, scoliosis, pain
5	F	14	*TEK* c.2740C > T/p.L914F	Sanger sequencing	VeM of right cheek, pain, headaches
6	F	3	*TEK* c.2690A > G/p.Y897C (germline)	Sanger sequencing	Multiple VLM of the cranium, subcutaneously on the head, thorax, and limbs
7	M	19	*PIK3CA* c.1633G > A/p.E545K	NGS	Sporadic AVM of left lower limb involving m. semimembranosus, movement limitation, pain
8	F	21	*PIK3CA* c.3140A > G/p.H1047R	NGS	VeM of the right foot, pain, inability to walk
9	M	17	*TEK* c.2740C > T/p.L914F	Sanger sequencing	VeM of right lower limb, genitals, movement limitations, pain, coagulopathy
10	F	17	*TEK* c.2753G > A/p.R918H	Sanger sequencing	VeM of right upper limb and shoulder, chronic coagulopathy, pain, DIC
11	F	37	*TEK* c.2752C > T/p.R918C	WES	VeM of left lower limb and pelvis, movement limitations, pain
12	F	22	*PIK3CA* c.1633G > A/p.E545K	NGS	VeM/hemangioma of left lower limb involving m. vast. intermed., inoperable, pain, movement limitations
13	M	9	*TEK* c.2740C > T/p.L914F	NGS	VeM of right lower limb (involving m. gluteus max., m. semitendinosus, m. vastus intermedius, m. semimembranosus, m. biceps femoris), inoperable, pain, movement limitations, chronic coagulopathy
14	F	14	*PIK3CA* c.3140A > G/p.H1047R	NGS	VeM of left lower lim assoc. with Klippel-Trenaunay syndrome, chronic coagulopathy
15	F	3	*TEK* c.3323_3324del/p.Y1108*	WES	VeM of the face forming from right ear to chin involving muscles of oral cavity and tongue
16	F	7	*TEK* c.2740C > T/p.L914F	Sanger sequencing	VeM of the left knee, intramuscular involvement, pain, movement limitations
17	F	2	*TEK* c.2740C > T/p.L914F	NGS	Generalized VeM of the neck, oro- and nasopharynx, back, chest wall, mediastinum, buttocks, thighs, knees, and left shin and foot
18	M	11	*TEK* c.2740C > T/p.L914F	Sanger sequencing	VeM of the right hand, pain, mobility impairment

Table 1. List of patients with basic characteristics and clinical symptoms, class of VM, results of molecular analysis, and sequencing methods used. *VM* vascular malformation, *VeM* venous malformation, *VLM* venolymphatic malformation, *AVM* arteriovenous malformation, *M* male, *F* female.

years with only temporary effects. After 5 months of alpelisib treatment, we observed a rapid size reduction (see Fig. 2) and significant improvements in both QoL measurements and coagulation markers.

Patient No. 8 came to our clinic with a VeM located in her right foot, which resulted in severe pain while walking. Within weeks after the introduction of alpelisib treatment, she was able to walk and soon even run without any limitations. The effect of the treatment on the VeM size reduction and structure regression is well documented on MRI of the right foot prior to and 3 months after alpelisib treatment (see Fig. 3).

The patient with Noonan syndrome (No. 3) and a somatic *TEK* mutation had a rapid but only partial effect of the single-agent alpelisib treatment; therefore, a MEK inhibitor was added to target *PTPN11* germline mutation, which may have contributed to the pathogenesis and thus to enhance the effect of alpelisib. However, very soon after the start of this double-agent therapy, the patient suffered from severe headaches and nausea, leading to the temporary discontinuation of both drugs. Within 2 weeks of discontinuation, we observed a rapid increase in the size of the malformation and D-dimer levels. Therefore, alpelisib treatment was restarted as monotherapy at a 50% dose reduction with good patient compliance and both clinical and laboratory efficacy in decreasing D-dimer levels.

The oldest patient was a 36-year-old woman who underwent a series of 25 surgeries and multiple nontargeted treatments for her VeM of a left lower limb starting in childhood before genetic testing discovered a pathogenic mutation of the *TEK* gene. After starting alpelisib treatment, we observed rapid clinical effects in QoL and size reduction of the lesions, which was later confirmed with MRI imaging.

Patient no. 12, with VeM of the left lower limb with muscular involvement, started the treatment due to potentially disabling surgery. Six months after the alpelisib administration, we achieved size reduction allowing safe total resection of the lesion without collateral damage to the healthy tissue. Six months after the procedure, the patient remains asymptomatic without any treatment (See Fig. 4).

Patient	Age at treatment initiation (years)	Indication for treatment[a] (P / M / C)	Duration of treatment[b] and initial dose	QoL	MRI (% of volume reduction comparing pre- and posttherapy imaging) (* Planar size reduction)	D-dimer level	Side effect	Compliance
1	7	P, M, C	24 months (150 mg/day)	Marked improvement of pain, lesion tenderness, and mobility after 1 month of the treatment	Stable	Marked improvement after 3 months of the treatment	None	Well tolerated
2	17	P, M, C	30 months (200 mg/day)	Marked improvement of pain, lesion tenderness, and mobility after 1 month of the treatment	*41% reduction after 6 months of the treatment	Marked improvement after 3 months of the treatment, elevation when treatment paused	IDDM, headache, menorrhagia, abdominal pain	Need to temporary pause the treatment due to IDDM
3	16	P, M, C	31 months (200 mg/day)	Marked improvement of pain, lesion tenderness, and mobility after 6 weeks of the treatment	20% reduction after 2 months of the treatment	Marked improvement after 6 months of the treatment, elevation when treatment paused	Headaches when combined with trametinib	Well tolerated as a single agent
4	8	P, M	30 months (200 mg/day)	Marked improvement of pain after 3 months of the treatment	41% reduction regression after 6 m of the treatment	Within normal range before the start of the treatment	Hypertension	Well tolerated
5	12	P, M, C	24 months (300 mg/day)	Marked improvement of pain and lesion tenderness after 3 months of the treatment	34% reduction after 8 months of the treatment	Marked improvement after 2 months of the treatment	Hair loss, nausea	Well tolerated in reduced dose
6	1	P, M, C	23 months (80 mg/day)	Marked improvement of pain and lesion tenderness after 3 months of the treatment	Progression of lesions after 6 months of the treatment, 23% reduction after 4 months of alpe/sirolimus combination	Marked improvement after 1 month of the treatment	None	Well tolerated
7	17	P, M	23 months (200 mg/day)	Marked improvement of pain and mobility after 1 month of the treatment, recurrent pain after one month of alpelisib pause	30% reduction after 6 months of the treatment	Within normal range before the start of the treatment	None	Well tolerated
8	19	P, M	Stopped after 9 months on patient request (200 mg/day)	Marked improvement of pain and mobility after 1 month of the treatment	61% reduction after 6 months of the treatment	Within normal range before the start of the treatment	Hyperglycemia on oral hypoglycemic agents, gastritis	Initially well tolerated, discontinued based on patient request
9	15	P, M, C	22 months (300 mg/day)	Marked improvement of pain and mobility after 1 month of the treatment	Stable after 6 months of the treatment, 25% reduction after 4 months of alpe/propranolol combination	Improvement after 6 months of the treatment	None	Well tolerated
10	15	P, M, C	18 months (300 mg/day)	Marked improvement of pain, lesion tenderness, and mobility after 1 month of the treatment	57% reduction after 5 months of the treatment, stationary/slight progression after 12 months of the treatment	Marked improvement after 1 month of the treatment	Hyperglycemia without the need for a specific medication	Well tolerated
11	35	P, M, C	30 months (300 mg/day)	Marked improvement of pain, lesion tenderness, and mobility after 1 month of the treatment	*11% reduction after 16 months of the treatment	Marked improvement after 3 months of the treatment	None	Well tolerated
12	20	P, M	Stopped after 6 months due to resection of the lesion (200 mg/day)	Marked pain reduction and mobility improvement	87% reduction after 3 months of the treatment, lesion operable	Within normal range before the start of the treatment	Headaches	Well tolerated

Continued

Patient	Age at treatment initiation (years)	Indication for treatment[a] (P / M / C)	Duration of treatment[b] and initial dose	QoL	MRI (% of volume reduction comparing pre- and posttherapy imaging) (* Planar size reduction)	D-dimer level	Side effect	Compliance
13	8	P, M, C	9 months (200 mg/day)	Marked improvement of pain, lesion tenderness, and mobility after 1 month of the treatment	Stable	Marked improvement after 1 month o the treatment	None	Well tolerated
14	13	P, M, C	14 months (200 mg/day)	Marked improvement of pain, lesion tenderness, and mobility after 3 months of the treatment	*45% reduction after 4 months of the treatment	Marked improvement after 1 month of the treatment	None	Well tolerated
15	2	P, C	12 months (100 mg/day)	Lesions discolored and less tender after 2 months of the treatment	19% reduction after 6 months of the treatment	Marked improvement after 3 months of the therapy	None	Well tolerated
16	6	P, M, C	10 months (150 mg/day)	Marked improvement of pain, lesion tenderness, and mobility after 1 month of the treatment	N/A at the time of publication	Marked improvement after 3 months of the treatment	None	Well tolerated
17	2	P, M, C	6 months (40 mg/day)	Improvement of pain, lesions discolorized, less tender after 3 months of the treatment	N/A at the time of publication	Marked improvement after 3 months of the treatment	None	Well tolerated
18	11	P, M, C	6 month (200 mg/day)	Marked improvement of pain and mobility after 3 months	5% reduction after 3 moonths of the treatment	Within normal range before the start of the treatment	None	Well tolerated

Table 2. List of patients with the treatment indication, duration of treatment, starting dose and the effect of the treatment on quality of life (QoL), size on MRI, and coagulation markers. [a]Indications for the treatment: *P* pain, *M* mobility impairment, *C* Coagulopathy. [b]Duration of treatment as of May 2023: *QoL* quality of life, *IDDM* insulin dependent diabetes mellitus, *N/A* not applicable.

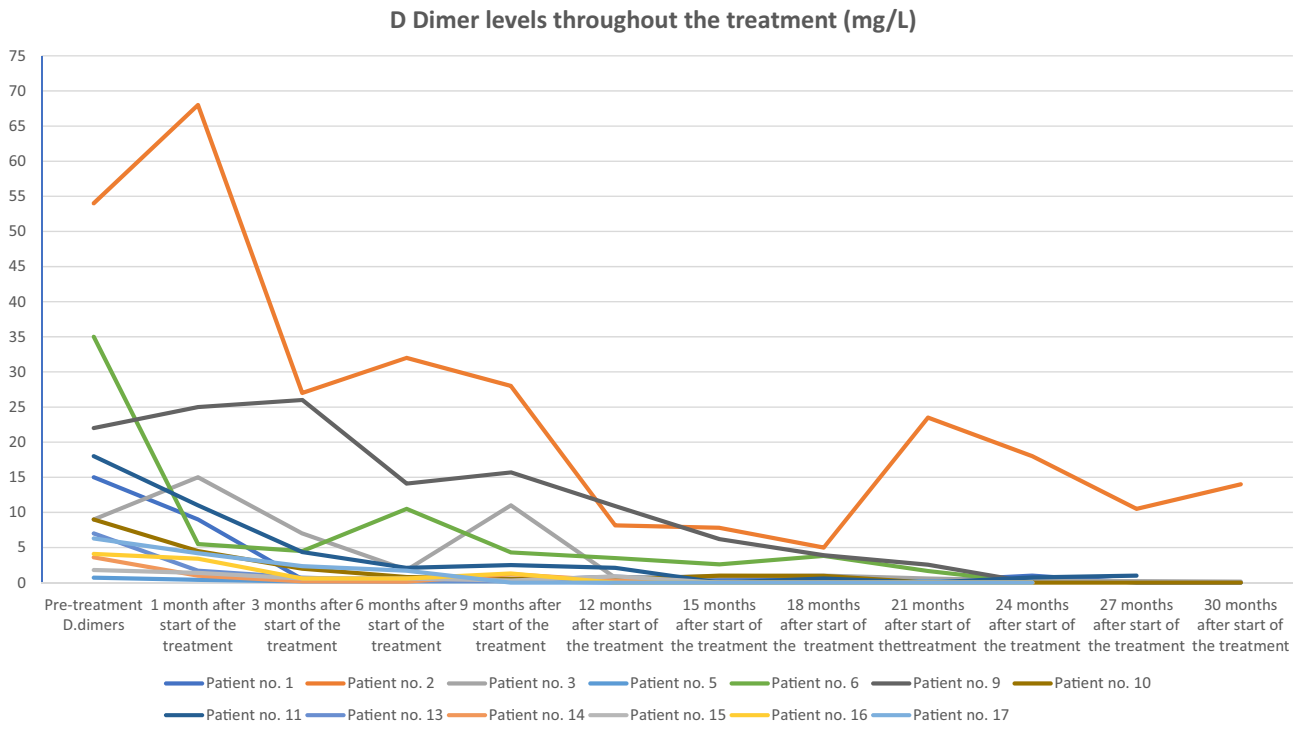

Figure 1. D-dimer levels throughout alpelisib treatment in patients whose levels were outside the normal range before treatment initiation.

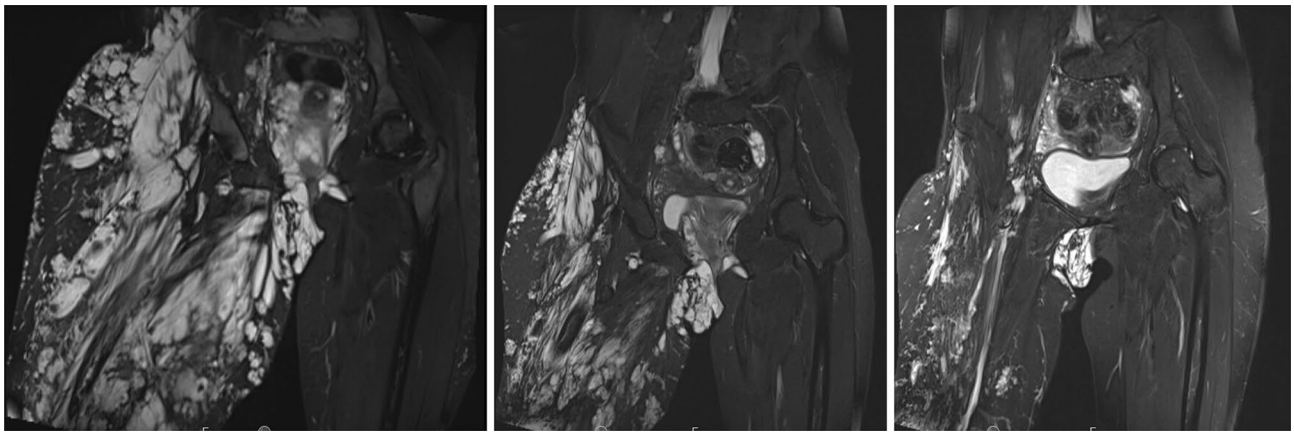

Figure 2. Patient number 2. MRI (T2 STIR cor. plane) of the pelvis and proximal thighs prior to (left), 6 months middle) and 12 months after alpelisib treatment (right).

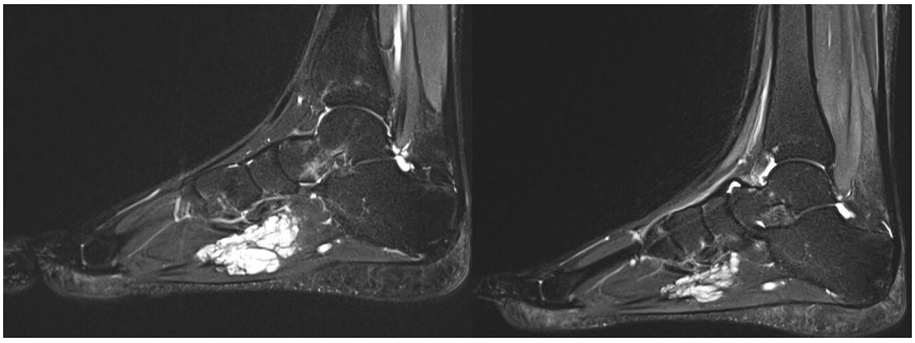

Figure 3. Patient number 8. MRI (T2 STIR sagit. plane) of the right foot prior (left) and 3 months after alpelisib treatment (right).

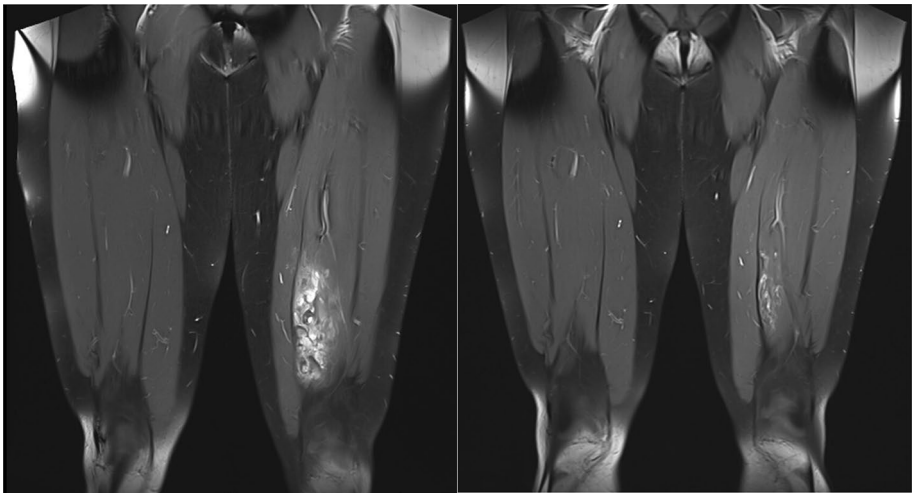

Figure 4. Patient number 12. MRI (T1 STIR cor. Plane) of the thighs prior (left) and 3 months after alpelisib treatment (right).

Discussion

The use of targeted therapeutics for the management of VMs represents a new off-label therapeutic strategy, which could be used as an addition to classic local treatment approaches following the paradigm of precision medicine. Drugs that are currently being used in this setting include mTOR inhibitors[17], PI3K inhibitors[11], AKT

inhibitors[18], MEK inhibitors[19], and VEGF inhibitors[7]; however, some of them show only limited efficacy, possibly because they are administered without prior knowledge of the driving gene mutation.

In this observational study, activating mutations in the *PIK3CA* and *TEK* genes were discovered in the lesions of 18 patients. Molecular analyses were performed using various sequencing methods ranging from Sanger sequencing to next-generation sequencing of targeted gene panels or WES. These different methods were selected based on current availability and the clinical picture suggesting a particular mutation in a given patient. The best diagnostic algorithm for the future, reflecting both economic and medical needs, remains to be established and is the subject of a future project.

Obtaining a representative piece of the lesion for subsequent analyses can become the first major obstacle due to the high risk of severe bleeding. A multidisciplinary team of specialists is of vital importance in facing such challenges. In patients with a difficult surgical approach, liquid biopsy could potentially help to discover driving mutations and may serve as a potential diagnostic marker; however, further investigation is needed[20].

The starting dose of alpelisib was selected based on BSA-modified dosing schemes for the treatment of patients with breast cancer for whom a single dose of 300 mg is recommended (calculated 173 mg/m^2 BSA). Our study used a 20% dose reduction (138.6 mg/m^2/day) with high efficacy. Thus, for patients with insufficient response, the dose can be increased. In addition, for one of our patients who suffered an adverse event, restarting the drug with a 50% reduction was sufficient to maintain efficacy with a response measurable clinically and in D-dimer levels. In patient with hyperglycemia grade 3 toxicity we observed regrowth of the lesion after alpelisib cessation, restarting drug in 25% of the original dose led to suboptimal clinical and D-dimer response, however, at 37% of the original dose there was a very good both clinical and laboratory effect with only low dose of insulin therapy. Management of alpelisib induced diabetes mellitus was recently described in case report by Pia Peris et al.[21].

To evaluate the therapeutic effect, patients underwent regular check-ups consisting of current history taking, clinical investigation, and laboratory testing. With the more recent patients, we also performed MRI scans before and 6 months after the start of the treatment. A measurable response was observed in 14 out of 18 patients on MRI, and a visual clinical response was observed in all patients, proving that alpelisib is highly effective. In all patients, we observed a striking effect on clinical symptomatology within the first few months of the treatment. Similar efficacy was reported by other authors describing their experience in a cohort of 19 patients and two case reports of patients with *PIK3CA*-related overgrowth syndrome (PROS)[11–13]. In our study, we also report the efficacy of alpelisib on VMs in patients without germline mutations but with somatic mutations, and moreover, this report describes alpelisib efficacy in the largest cohort of patients with somatic mutations in the *TEK* gene so far, thus following up on work of Remy et al.[15].

When compared the radiological response in patients with *TEK* and *PIK3CA* mutations, both the average volume reduction and median value were higher in patients harboring *PIK3CA* mutation, however larger group would be needed for stronger results. The response in QoL parameters and coagulation markers were similar for both groups.

In patients who originally suffered from chronic coagulopathy, alpelisib treatment resulted in a decrease and even normalization of D-dimer levels, which seems to be a very useful marker of treatment success. Furthermore, we observed pain relief with quality-of-life improvement as the most important therapeutic goal. We have not observed any signs of cumulative toxicity comparing the first and second 6 months of drug exposure thus far.

After treatment discontinuation in two patients, we observed both clinical and paraclinical deterioration with a rapid rise of D-dimer levels, suggesting the need for prolonged treatment exposure. However, the optimal treatment duration and minimal effective dose remain unknown, as does the long-term toxicity profile in young patients. Different dosing and scheduling schemes have yet to be tested. Another open question is identifying the so-called "best achievable response" and reintroducing different local therapeutic options. Subjects who achieve size reduction after alpelisib treatment can be recommended for local treatments with a much lower risk of mutilation or complications such as bleeding or anatomical structure destruction. In our cohort, one patient (No. 12) experienced a 87% volume reduction of initially unresectable lesions on alpelisib, and the residual lesions were resected without bleeding or functional deficits of the thigh.

This prospective observational study contributes informations about a new treatment option that is well tolerated and shows objective responses with highly appreciated quality of life improvement.

Methods

All patients or their legal guardians signed informed consent with molecular genetic testing and off-label treatment using alpelisib. A total of 18 patients with large, debilitating or opioid requiring VMs with one or more major systemic complications (e.g., chronic systemic consumption coagulopathy, major organ involvement or multiple bone and joint involvement, scoliosis) underwent molecular genetic analysis using either whole-exome sequencing (WES), a targeted next-generation sequencing (NGS) panel, or direct sequencing of *TEK* exon 17 by Sanger's method. The analyses were performed on lesion biopsy specimens. DNA was extracted from FFPE tissue using QIAmp FFPE Tissue Kit (Qiagen, Germany) and treated with NEBNext FFPE DNA Repair Mix (New England Biolabs, MA, USA). For WES, both lesion and matched normal genomic DNA were used. Libraries were prepared using TruSeq DNA Exome (Illumina, CA, USA) according to the manufacturer's instructions and sequenced on the NextSeq500 platform using NextSeq 500/550 Mid Output Kit v2.5 (150 Cycles) (Illumina). Libraries for targeted NGS were prepared using QIAseq Targeted DNA Panel—Human Actionable Solid Tumor (Qiagen) according to the manufacturer's instructions and sequenced on the NextSeq500 platform using NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles) (Illumina). For direct sequencing of exon 17 of the *TEK* gene, custom-made PCR primers (IDT, NJ, USA) were used to amplify the target sequence (F: 5′-CCTGGGTGGTGT TGCTAGAT-3′, R: 5′-AGAGGGAACTCCACAGGAAAG-3′). Sequencing analysis of a purified and labeled PCR

product was performed on the ABI 3130xl device (ThermoFisher Scientific, MA, USA). Patients with confirmed mutations in *PIK3CA* and *TEK* were recruited for alpelisib treatment and efficacy analysis.

All patients included in our study were Caucasian. Prior to the start of alpelisib treatment, a thorough patient history, clinical examination, baseline imaging, and laboratory tests, including D-dimer levels, were obtained. Initial clinical symptoms with basic demographic data, class of VM according to ISSVA (International society for the study of vascular anomalies) and discovered mutation types are summarized in Table 1.

Based on molecular findings and clinical symptoms, all 18 patients began orally administered alpelisib treatment. The dosage used for our patients was derived from the breast cancer guidelines and individually adjusted to body surface area (BSA) and varied from 50 to 300 mg per dose. The average starting dose was 138.6 mg/m^2/day. As our center was not part of the EPIK-P1 study, the dosing of alpelisib in our patients differs from the dosing schedule in EPIK-P1, which led to the FDA approval of alpelisib for *PIK3CA*-related Overgrowth Spectrum patients in April 2022[22].

Regular check-ups were performed to evaluate the efficacy of the treatment. Each patient was examined in an outpatient clinic with laboratory and clinical examinations every 4 weeks and imaging, mostly MRI scan, every 3 months ± 2 weeks.

Quality of life (QoL) was assessed with a revised patient control outcome questionnaire which included an assessment of both pain and functional impairment resulting in a four-step semiquantitative scale: worse, stable, improved, and marked improvement.

Adverse events were classified according to CTCAE v5.0.

Discontinuation of the alpelisib treatment was allowed at the patient´s request due to adverse events and at the discretion of the treating physician.

All methods were carried out in accordance with relevant guidelines and regulations.

Conclusion

Novel PI3K inhibitors such as alpelisib represent a promising therapeutic option for patients with confirmed *TEK* or *PIK3CA* gene alterations when local treatment methods have only partial or temporary effect or when the lesion is inoperable because of its location. This prospective observational study contributes information about this new treatment option, which is well tolerated documenting only 1 grade 3 toxicity, objective responses, and highly appreciated quality of life improvements.

Several questions still need to be answered, as this is an off-label, agnostic, and experimental treatment. Although we did not observe any side effect requiring permanent treatment discontinuation, long-term toxicity remains to be established. The optimal treatment duration, dosing, and scheduling are also a matter of further investigation, as well as the optimal position of this systemic treatment in the complex management of vascular malformations.

Data availability

The data that support the findings of this study are available from the corresponding author, [P. Mudry], upon reasonable request. Sequencing data are available from: https://www.ebi.ac.uk/ena/browser/view/PRJEB53413, accession number PRJEB53413.

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Author contributions
Conceptualization: M.S., P.P., P.M., J.S.; Methodology: P.P., O.S., H.P.; Investigation: R.F., B.P., J.S.k., A.S., J.B., L.J., O.K., M.M., L.P.; Data curation: R.F., B.P., J.S.k., A.S., J.B., L.J., O.K., M.M., L.P.; Resources: M.S., P.P., H.P., P.J., P.M.; Writing—original draft: M.S.; Writing–Review and Editing: P.P., P.M., J.S.; Supervision: O.S., P.J., P.M., J.S. All authors approved the final manuscript.

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Competing interests
The authors declare no competing interests.

Additional information
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