Molecular testing of rhabdomyosarcoma in clinical trials to improve risk stratification and outcome

Hettmer, Simone; Linardic, Corinne M.; Kelsey, Anna; Rudzinski, Erin R.; Vokuhl, Christian; Selfe, Joanna; Ruhen, Olivia; Shern, Jack F.; Khan, Javed; Kovach, Alexander R.; Lupo, Philip J.; Gatz, Susanne A.; Schäfer, Beat W.; Volchenboum, Samuel; Minard-Colin, Véronique; Koscielniak, Ewa; Hawkins, Douglas S.; Bisogno, Gianni; Sparber-Sauer, Monika; Venkatramani, Rajkumar

DOI:
10.1016/j.ejca.2022.05.036

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Hettmer, S, Linardic, CM, Kelsey, A, Rudzinski, ER, Vokuhl, C, Selfe, J, Ruhen, O, Shern, JF, Khan, J, Kovach, AR, Lupo, PJ, Gatz, SA, Schäfer, BW, Volchenboum, S, Minard-Colin, V, Koscielniak, E, Hawkins, DS, Bisogno, G, Sparber-Sauer, M, Venkatramani, R, Merks, JHM & Shipley, JM 2022, 'Molecular testing of rhabdomyosarcoma in clinical trials to improve risk stratification and outcome: A consensus view from European paediatric Soft tissue sarcoma Study Group, Children's Oncology Group and Cooperative Weichteilsarkom-Studiengruppe', *European Journal of Cancer* , vol. 172, pp. 367-386. https://doi.org/10.1016/j.ejca.2022.05.036

European Journal of Cancer 172 (2022) 367–386

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Review

Molecular testing of rhabdomyosarcoma in clinical trials to improve risk stratification and outcome: A consensus view from European paediatric Soft tissue sarcoma Study Group, Children's Oncology Group and Cooperative Weichteilsarkom-Studiengruppe

Simone Hettmer [a,1], Corinne M. Linardic [b,c,1], Anna Kelsey [d], Erin R. Rudzinski [e,f], Christian Vokuhl [g], Joanna Selfe [h], Olivia Ruhen [h], Jack F. Shern [i,j], Javed Khan [i], Alexander R. Kovach [c], Philip J. Lupo [k], Susanne A. Gatz [l], Beat W. Schäfer [m], Samuel Volchenboum [n], Véronique Minard-Colin [o], Ewa Koscielniak [p,q], Douglas S. Hawkins [r], Gianni Bisogno [s], Monika Sparber-Sauer [p,q], Rajkumar Venkatramani [t], Johannes H.M. Merks [u], Janet Shipley [h,*]

[a] *Division of Pediatric Hematology and Oncology, Department of Pediatric and Adolescent Medicine, University Medical Center Freiburg, University of Freiburg, Germany*
[b] *Department of Pediatrics, Duke University School of Medicine, Durham, NC, USA*
[c] *Department of Pharmacology and Cancer Biology; Duke University of Medicine, Durham, NC, USA*
[d] *Department of Paediatric Histopathology, Royal Manchester Children's Hospital, Manchester Foundation Trust, Manchester, UK*
[e] *Section of Hematology-Oncology, Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA*
[f] *Department of Laboratories, Seattle Children's Hospital, Seattle, WA, USA*
[g] *Section of Pediatric Pathology, Department of Pathology, University Hospital Bonn, Germany*
[h] *Sarcoma Molecular Pathology Team, Divisions of Molecular Pathology and Cancer Therapeutics, The Institute of Cancer Research, London, UK*
[i] *Genetics Branch, Oncogenomics Section, Center for Cancer Research, National Institutes of Health, Bethesda, MD, USA*
[j] *Pediatric Oncology Branch, Center for Cancer Research, National Institutes of Health, Bethesda, MD, USA*
[k] *Department of Pediatrics, Section of Hematology-Oncology, Baylor College of Medicine, Houston, TX 77030, USA*
[l] *Institute of Cancer and Genomic Sciences, Cancer Research UK Clinical Trials Unit (CRCTU), University of Birmingham, Birmingham, UK*
[m] *Department of Oncology and Children's Research Center, University Children's Hospital Zurich, Zurich, Switzerland*
[n] *Department of Pediatrics, University of Chicago, USA*
[o] *Department of Pediatrics, Institut Gustave-Roussy, 94805 Villejuif Cedex, France*
[p] *Klinikum der Landeshauptstadt Stuttgart GKAöR, Olgahospital, Stuttgart Cancer Center, Zentrum für Kinder-, Jugend- und Frauenmedizin, Pädiatrie 5 (Pädiatrische Onkologie, Hämatologie, Immunologie), Stuttgart, Germany*
[q] *Medizinische Fakultät, University of Tübingen, Germany*

* *Corresponding author*: The Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5NG. UK
E-mail address: janet.shipley@icr.ac.uk (J. Shipley).
[1] These authors contributed equally.

https://doi.org/10.1016/j.ejca.2022.05.036

[r] Seattle Children's Hospital, University of Washington, Fred Hutchinson Cancer Research Center, Seattle, WA, USA
[s] Hematology Oncology Division, Department of Women's and Children's Health, University of Padova, Padua, Italy
[t] Department of Pediatrics, Texas Children's Hospital, Baylor College of Medicine, Houston, TX, USA
[u] Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

Received 3 February 2022; received in revised form 27 April 2022; accepted 22 May 2022
Available online 12 July 2022

KEYWORDS
Rhabdomyosarcoma;
paediatric;
adolescent;
young adults;
germ line and somatic genetics;
molecular biomarkers;
gene signatures;
molecular targets

Abstract Rhabdomyosarcomas (RMSs) are the most common soft tissue sarcomas in children/adolescents less than 18 years of age with an annual incidence of 1–2/million. Inter/intra-tumour heterogeneity raise challenges in clinical, pathological and biological research studies. Risk stratification in European and North American clinical trials previously relied on clinico-pathological features, but now, incorporates *PAX3/7-FOXO1*-fusion gene status in the place of alveolar histology. International working groups propose a coordinated approach through the INternational Soft Tissue SaRcoma ConsorTium to evaluate the specific genetic abnormalities and generate and integrate molecular and clinical data related to patients with RMS across different trial settings. We review relevant data and present a consensus view on what molecular features should be assessed. In particular, we recommend the assessment of the *MYOD1-LR122R* mutation for risk escalation, as it has been associated with poor outcomes in spindle/sclerosing RMS and rare RMS with classic embryonal histopathology. The prospective analyses of rare fusion genes beyond *PAX3/7-FOXO1* will generate new data linked to outcomes and assessment of *TP53* mutations and *CDK4* amplification may confirm their prognostic value. Pathogenic/likely pathogenic germline variants in *TP53* and other cancer predisposition genes should also be assessed. DNA/RNA profiling of tumours at diagnosis/relapse and serial analyses of plasma samples is recommended where possible to validate potential molecular biomarkers, identify new biomarkers and assess how liquid biopsy analyses can have the greatest benefit. Together with the development of new molecularly-derived therapeutic strategies that we review, a synchronised international approach is expected to enhance progress towards improved treatment assignment, management and outcomes for patients with RMS.

1. Introduction

Rhabdomyosarcomas (RMSs) comprise a heterogeneous group of high-grade malignant neoplasms with a propensity for myogenic differentiation. While RMS may be diagnosed at any age, it is more common in children, adolescents and young adults and accounts for approximately half of all soft tissue sarcomas diagnosed in the paediatric age group [1]. With the development and refinement of multimodal treatment regimens, survival has improved substantially for many children with RMS. However, the survival of those diagnosed with widely metastatic or relapsed disease continues to be very low [2,3].

The World Health Organisation recognises three RMS histotypes that arise in young people, i.e. embryonal, alveolar and spindle cell sclerosing RMS [4]. These pose distinct challenges in diagnostic classification and treatment. Alveolar and embryonal RMSs are the most common histotypes in children and adolescents. In 80% of cases with alveolar histology, balanced chromosomal translocations involving chromosomes 2 or 1 and chromosome 13 result in expression of the fusion oncoproteins PAX3-FOXO1 or PAX7-FOXO1, respectively, that drive the malignant phenotypes of these tumours [5–7]. Pathological, clinical and molecular diversity is noted in the embryonal subtype of RMS with genetic aberrations including frequent chromosomal gains (chromosomes 2, 8 and 13), mutations of genes such as those in the RAS pathway and specific regions of the loss of heterozygosity and imprinting [8,9].

We strongly believe that RMS biology should directly inform clinical trial design. In recent years, insights into the genetics and molecular biology of RMS have provided much-needed opportunities to improve disease classification, risk stratification, assessment of treatment response and opportunities for targeted therapies. Cooperative groups in Europe and North America have begun to enhance the risk-stratification algorithms by incorporating molecular characteristics, such as *PAX3/*

7-FOXO1-fusion gene status [10–14]. A number of other biomarkers, including specific gene mutations/amplifications and gene expression signatures, are awaiting prospective validation. The collection of biomaterials as part of clinical trials is critically important to correlate molecular characteristics with clinical parameters and response to treatment.

The translation of preclinical findings into clinical trials and, ultimately, standard-of-care recommendations will be more robust and delivered substantially faster if research priorities are coordinated by cooperative groups. The INternational Soft Tissue SaRcoma ConsorTium (INSTRuCT) (https://commons.cri.uchicago.edu/instruct/), a cooperation of the European paediatric Soft tissue sarcoma Study Group (EpSSG) (created in 2004 by the merging of the International Society of Pediatric Oncology – Malignant Mesenchymal Tumour Committee [SIOP-MMT], and the Associazione Italiana di Ematologia e Oncologia Paediatrica Soft Tissue Sarcoma Committee), the Children's Oncology Group (COG) and the Cooperative Weichteilsarkom Studiengruppe (CWS) offer a platform to harmonise prospective molecular testing and coordinate investigations as part of large clinical trials [15]. This manuscript builds on a recent review of childhood RMS pathology and the current use of immunohistochemistry and fusion gene status [16]. Here we review and recommend analyses of genetic aberrations, including fusion genes, somatic and germline genetic aberrations and gene expression signatures in the context of trials to determine their possible prognostic, predictive and associated therapeutic implications. We also discuss the sample collection and molecular analyses that we believe should be undertaken in trials for further future benefits.

2. Potential for genetic biomarkers

2.1. Fusion genes

2.1.1. *PAX3/7-FOXO1*-fusion genes

Both the t (2; 13) (q35; q14) and less frequent t (1; 13) (p36; q14) translocations are associated with the majority of the alveolar subtype of RMS and result in fusion oncoproteins consisting of the N-terminal DNA-binding domains of PAX3 or PAX7 fused to the C-terminal transactivation domain of FOXO1 [5–7]. The presence of the *PAX3-FOXO1*-fusion gene has been shown to be associated with significant negative prognostic value in RMS in several studies and is more frequent in adolescents than younger patients [11,13,14,17]. Importantly, gene expression profiling also demonstrated that *PAX3/7-FOXO1*-negative alveolar histology tumours were similar to embryonal histology tumours [10,14]. These observations, previously reviewed in the context changing histological criteria over time and between working groups [16], have stimulated modifications in RMS risk stratification that reduces the intensity of therapy for patients with *PAX3/7-FOXO1*-negative, localised alveolar histology tumours [12,18]. Modified risk stratification is now being evaluated prospectively in the COG (ARST1431) and EpSSG Frontline and Relapsed-RhabdoMyoSarcoma (FaR-RMS) studies.

The partner gene fused to *FOXO1* may hold additional prognostic significance, with *PAX7-FOXO1* tumours possibly having superior overall survival compared to *PAX3:FOXO1*-translocated tumours [11,13,19,20]. However, a study by Stegmaier et al. did not report significant differences in survival between patients with *PAX7-FOXO1* versus *PAX3-FOXO1*-translocated localised or metastatic tumours (n = 101) [21,22]. In contrast, a recent study of 243 patients with known *PAX3/7-FOXO1* status supported a superior outcome in cases involving the rearrangement of PAX7 versus PAX3 [23]. Prospective assessment in the COG (ARST1431) and EpSSG (FaR-RMS) trials, with potential to jointly assess, will provide a more definitive evaluation.

The current diagnostic algorithms used by the EpSSG, COG and CWS- working groups to identify RMS gene fusions in the context of pathological diagnosis have been recently reviewed [16]. The immunohistochemical detection of downstream targets of the PAX3-FOXO1-fusion proteins as well as detection of the PAX3/7-FOXO1 protein itself may be useful in aiding diagnosis but require further validation [24–26].

2.1.2. *Rare variant fusion genes*

Alternative *PAX* gene translocations have been identified in a subset of *PAX3/7-FOXO1*-fusion negative alveolar histology RMS, including *PAX3-AFX (FOXO4)*, *PAX3-NCOA1*, *PAX3-NCOA2* and *PAX3-INO80D* [23,27–29]. Barr et al. also reported two alveolar cases with *PAX3* and *PAX7 f*usions in which the translocation partner remained unknown [27]. Non-*PAX* gene translocations are associated with spindle cell RMS where the genes encoding the serum response factor SRF and the transcription factors TEAD1 and VGLL2 fuse with the nuclear receptor coactivator NCOA2 [30,31]. The transcription coactivator CITED2 may also fuse to VGLL2 [30]. Of note, spindle cell RMS with recurrent *NCOA2* or *VGLL2* gene fusions have been associated with very young age at diagnosis, localised disease and excellent outcomes [30–33], although high-grade transformation has been described in four cases with *VGLL2* gene fusions [34]. A group of tumours with the extensive cytodifferentiation resembling RMS but with infiltrative borders and nuclear atypia was also recently shown to harbour *SRF* fusions with *NCOA1* or *FOXO1* [35]. Finally, although more common in adults, a primary osseous spindle cell RMS subtype has been recently described [36–38]. The limited number of cases with EWSR1/FUS-TFCP2 rearrangements tends to show a hybrid spindled and epithelioid morphology with a

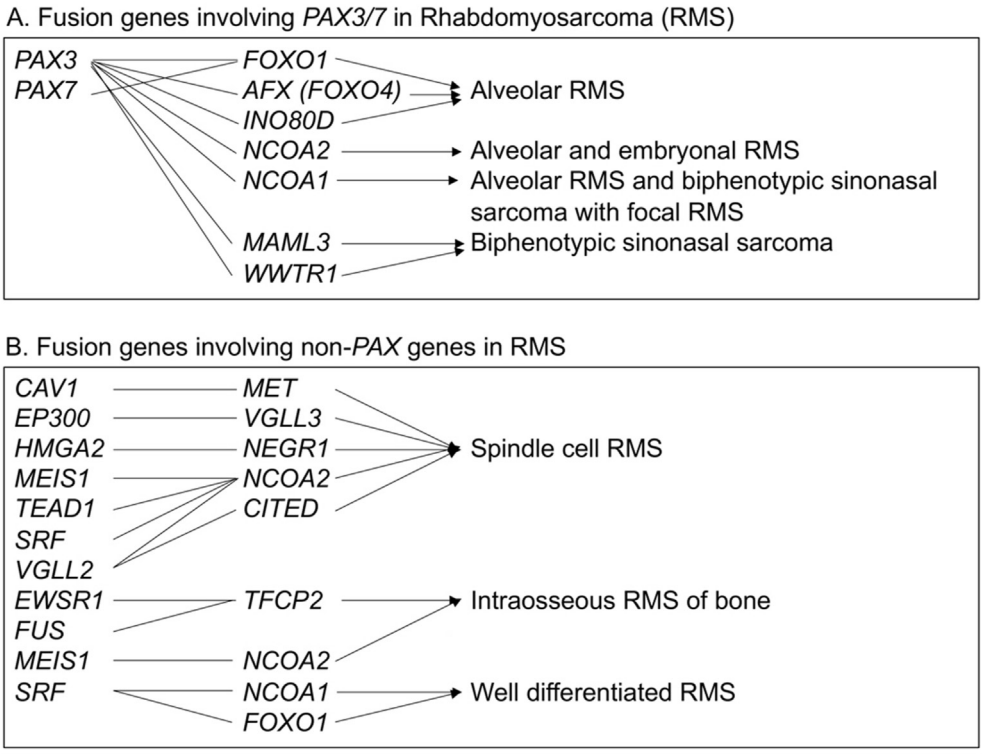

Fig. 1. **Fusion genes associated with histopathological subtypes of rhabdomyosarcomas (RMS).** (**a**) Fusion genes involving *PAX3/7* and (**b**) Fusion genes involving genes other than *PAX* genes.

frequent expression of cytokeratins and ALK and have poor outcomes [39], while those with NCOA2-MEIS1 fusions show a primitive, fascicular pattern. Additionally, new fusion genes have recently described in spindle cell RMS, including EP300-VGLL3, CAV1-MET and HMGA2-NEGR1 [40]. More data on all of these rare subtypes, summarised in Fig. 1, are needed to determine their molecular function and range in clinical characteristics and prognosis. Furthermore, the application of increasingly powerful and accessible sequencing technologies, including RNA sequencing, may facilitate biological understanding and the identification of additional rare fusion genes.

2.2. Genetic predisposition to RMS

Having a cancer predisposition syndrome is one of the strongest risk factors for developing RMS. Pathogenic/likely pathogenic (P/LP) germline variants in cancer predisposition genes in RMS development are summarised in Table 1. The genetic syndromes that have been implicated in susceptibility to RMS include (i) Li-Fraumeni (frequently associated with pathogenic *TP53* germline variants) [41]; (ii) neurofibromatosis type 1 (associated with pathogenic variants in *NF1*) [42]; (iii) nevoid basal cell carcinoma syndrome (frequently associated with pathogenic *PTCH1* and *SUFU* germline variants) [43]; (iv) DICER1 [44]; (v) Costello (frequently associated with pathogenic *HRAS* germline variants) [45]; (vi) Noonan (a RASopathy linked to pathogenic variants in several germline genes including *CBL*) [46]; (vii) Beckwith-Wiedemann (associated with abnormal regulation of genes encoded by two imprinting centres at 11p15) [47–49] (see Section 2.2.2) (viii) Nijmegen Breakage Syndrome with mutations in *NBN* [50] and (ix) Rubinstein Taybi syndrome with mutations in *CREBBP* and *EP300* [51].

The advent of large-scale whole-exome and genome germline sequencing has facilitated a comprehensive approach to evaluating P/LP variants in cancer predisposition genes among children, adolescents and young adults diagnosed with RMS. Five studies have, thus far, been published evaluating a spectrum of cancer predisposition genes (ranging from 70 to 204 genes) in patients with RMS (total 1207 patients; 21 to 615 patients per study) < 29 years of age (Table 1). The reported prevalence of P/LP variants was 7–17% [52–56]. True heritability of RMS may be even higher and caused by variants in genes not yet recognised as relevant cancer predisposition genes, rare variants and/or interactions between variants [57]. Parallel tumour/germline sequencing studies and subsequent functional investigations are needed to further delineate the landscape of pathogenic germline variants that contribute to the RMS development.

The most frequent P/LP germline variants identified in patients with young-onset RMS were detected in *TP53*, *NF1*, and *BRCA2* (Table 1). Most P/LP variants

Table 1
Cancer predisposition genes (CPGs) with pathogenic/likely pathogenic (P/LP)[a] variants detected in children, adolescents and adults diagnosed with RMS.

CPG	HGNC[b] approved name	OMIM[c]	Zhang et al. 2015[d] 43 probands < 20 yo 7% P/LP CPGs	Gröbner et al. 2018[e] 21 probands < 25yo 14% P/LP CPGs	Akhavanfard et al. 2020[f] 134 probands < 29 yo 7% P/LP CPGs	Li et al. 2020[g] 615 probands < 25 yo 8% P/LP CPGs	Kim et al. 2020[h] 394 probands < 25 yo 17% P/LP CPGs
TP53	Tumour protein p53	191170	2	1	1	11	6
NF1	Neurofibromin 1	613113	0	1	2	9	4
BRCA2[i]	Breast cancer 2, early onset	600185	1	1	1	6	2
GBA	Glucosylceramidase beta	606463	Na	0	0	na	7
MUTYH	MutY homologue	604933	0	0	0	na	7
ATM	ATM serine/ threonine kinase	607585	0	0	1	na	4
DICER1	Dicer 1, ribonuclease type III	606241	0	0	1	2	2
ERCC2	ERCC excision repair 2, TFIIH Core Complex Helicase Subunit	126340	0	0	0	na	5
HRAS	HRas proto-oncogene, GTPase	190020	0	0	0	5	0
MMR genes	Mismatch repair genes	609309/600678/ 600259/120436	0	0	0	3	2
SBDS	SBDS ribosome maturation factor	607444	Na	0	0	na	5
RECQL4[j]	RecQ protein-like 4	603780	0	0	0	3	1
BLM[j]	Bloom syndrome, RecQ helicase-like	210900	0	0	0	2	1
CBL	Cbl proto-oncogene	165360	0	0	0	2	1
SDHx	Succinate dehydrogenase complex, subunit A/ B/C/D	600857/185470/ 602413/602690	0	0	0	3	0
TRIM37	Tripartite motif containing 37	605073	Na	0	0	na	3
ABCB11	ATP binding cassette subfamily B member 11	603201	Na	0	0	na	2
BUB1B[j]	BUB1 mitotic checkpoint serine/ threonine kinase B	602860	Na	0	0	1	1
CHEK2	Checkpoint kinase 2	604373	Na	0	1	0	1
COL7A1	Collagen type VII alpha 1 chain	120120	Na	0	0	na	2
SMARCA4	SWI/SNF-related, matrix-associated, actin-dependent	603254	0	0	1	0	1
ALK	ALK receptor tyrosine kinase	105590	0	0	0	1	0
ATR	ATR serine/ threonine kinase	601215	Na	na	1	na	0
BRCA1	Breast cancer 2, early onset	113705	0	0	0	1	0
CDKN1C	Cyclin-dependent kinase inhibitor 1C	600856	0	0	0	0	1
DOCK8	Dedicator of cytokinesis 8	611432	Na	0	0	na	1

(*continued on next page*)

Table 1 (*continued*)

CPG	HGNC[b] approved name	OMIM[c]	Zhang et al. 2015[d] 43 probands < 20 yo 7% P/LP CPGs	Gröbner et al. 2018[e] 21 probands < 25yo 14% P/LP CPGs	Akhavanfard et al. 2020[f] 134 probands < 29 yo 7% P/LP CPGs	Li et al. 2020[g] 615 probands < 25 yo 8% P/LP CPGs	Kim et al. 2020[h] 394 probands < 25 yo 17% P/LP CPGs
ERCC5	ERCC excision repair 5, endonuclease	133530	0	0	0	na	1
FANCA	FA complementation group A	607139	0	0	0	na	1
FANCC	FA complementation group C	613899	0	0	0	na	1
FGFR4	Fibroblast growth factor receptor 4	134935	Na	na	na	na	1
FH	Fumarate hydratase	136850	0	0	0	0	1
PTCH1	Patched 1	601309	0	0	0	1	0
PTEN	Phosphatase and tensin homologue	158350	0	0	0	1	0
RET	Ret proto-oncogene	164761	0	0	0	0	1
SERPINA1	Serpin family A member 1	107400	Na	0	0	na	1
TRIP13[j]	Thyroid hormone receptor interactor 13	604507	Na	na	na	1	0

Abbreviations: na, not analysed; P/LP, pathogenic/likely pathogenic.
[a] Class 4/5 according to ACMG guidelines.
[b] HUGO Gene Nomenclature Committee.
[c] All variants detected in autosomal recessive CPGs were mono-allelic.
[d] (Likely) pathogenic variants in 89 CPGs among 43 patients diagnosed with RMS <20 years of age.
[e] (Likely) pathogenic variants in 162 CPGs among 21 patients diagnosed with RMS <25 years of age.
[f] (Likely) pathogenic variants in 204 CPGs among 134 patients diagnosed with RMS under 29 years of age.
[g] (Likely) pathogenic variants in 70 CPGs among 615 patients diagnosed with RMS <25 years of age.
[h] (Likely) pathogenic variants in 130 CPGs among 394 patients diagnosed with RMS <25 years of age.
[i] P/LP *BRCA2* variants detected by Li *et al.* were significantly enriched in RMS cases compared to controls.
[j] P/LP variants in *RECQL4*, *BLM*, *BUB1B*, *TRIP13* detected by Li *et al.* were not significantly enriched in RMS cases compared to controls.

detected in autosomal recessive cancer predisposition genes were mono-allelic [52–56]. Kim *et al.* reported two patients with embryonal RMS and biallelic germline variants in *ERCC2*, as well as one patient with embryonal RMS and biallelic germline variants in *BRCA2* [54]. Relatively frequent germline variants in *BRCA2*, Fanconi anaemia and mismatch repair genes were unexpected [53,56–59]. Li *et al.* noted that only *BRAC2* variants were significantly enriched in RMS cases compared to controls, and no autosomal recessive variants were significantly higher in patients with RMS [55]. Additional studies are needed to fully evaluate the role of these genes on RMS susceptibility.

Germline sequencing efforts in RMS also indicated that the age at diagnosis and RMS histotype may point towards a P/LP germline background [54,55]. P/LP variants in cancer predisposition genes were detected more frequently in children diagnosed with RMS at an earlier age (i.e. three years of age or younger) [55,60]. The majority of RMS in the context of cancer predisposition syndromes appears to be of the embryonal subtype [55]. This is consistent with the observation that patients with embryonal RMS have higher rates of family members with a history of cancer and higher frequency of secondary primary neoplasms than those with alveolar histology [61,62], although future assessment of predisposition should clarify PAX3/7-FOXO1 status and other somatic genetic changes.

Circumstantial evidence has linked P/LP germline variants in cancer predisposition genes to specific RMS phenotypes: *TP53* P/LP germline variants were reported in 11 of 15 children with diffusely anaplastic RMS and young age at diagnosis [60,63]. *NF1* P/LP germline variants were associated with urogenital primary tumours and young age at diagnosis [64,65]. *DICER1* P/LP germline variants were linked to primary tumours in the female urogenital tract [44]. *PTCH1* and *SUFU* P/LP germline variants were observed in patients with highly differentiated, rhabdomyoma-like RMS [43]. Around 50% of RMS with *TP53* germ line variants were

located in the head and neck (n = 20) in two studies [63,66]. It will be important to further explore germline genotype–phenotype correlations to facilitate recognition of P/LP germline variants in children, adolescents and young adults diagnosed with RMS. With respect to the association of germline cancer predisposition variant with outcome, Kim *et al.* reported that P/LP germline variants was not associated with outcome, however, this study was limited by small numbers and only included patients at intermediate-risk [54]. Therefore, further investigation in the association of P/LP germline variants with the toxicity of treatment, predisposition to second malignancies and outcome are warranted and may provide further risk stratification for therapy.

2.3. Loss of imprinting

The disproportionate occurrence of RMS in children with certain cancer predisposition syndromes [55] includes those with the abnormal regulation of genes encoded by two imprinting centres on chromosome band 11p15. Genomic imprinting is an epigenetic control mechanism whereby gene expression (most often a cluster of genes) occurs from only one of the maternal or paternal alleles. Older nomenclature commonly referred to 'maternal or paternally imprinted genes,' but it is more accurate to state whether a gene is 'maternally or paternally expressed or repressed' [67]. Based on contemporary genomic studies, there are at least 200 imprinted genes in the human genome [68].

Critical to proper genomic imprinting is the 'imprinting control region,' [69]. The imprinting control region is rich in CpG (cytidine-phosphate-guanosine) repeats [70] which are differentially methylated between the maternal and paternal alleles. Methylation can drive two different cis-regulatory mechanisms. First methylation can alter access to the DNA of architectural proteins, such as the loss of CTCF binding [71], leading to enhancer hijacking and increased expression of Insulin Growth Factor 2 (IGF2) due to the altered chromatin looping (Fig. 2). Second, methylation can inhibit the expression of a locus-specific long non-coding RNA (lncRNA) that modifies chromatin structure and accessibility, suppressing downstream genes. Loss of imprinting (LOI) in cancers can lead to inappropriate expression levels of growth-control genes that should be maternally or paternally expressed or repressed. The most well-known genomic locus affected by LOI and LOH is the locus at chromosome 11p15.5 that is frequently involved in RMS (Fig. 2).

The 11p15.5 locus contains two imprinting centres, IGF2/H19 and CDKN1C/KCNQ1OT1, and has been shown to be aberrantly methylated in a variety of cancers [72]. LOI of the paternal allele at the IGF2/H19 locus due to hypomethylation leads to the gain of CTCF binding to an insulator, that leads to increased expression of the lncRNA H19, that repressed the expression of IGF2, resulting in growth restriction syndromes like Russell-Silver syndrome (no increased cancer risk) [73]. On the other hand, hypermethylation of the same insulator at the IGF2/H19 locus due to hypermethylation leads to inappropriate overexpression of IGF2 and has been implicated in a number of cancers including Wilms tumour [74,75] (Fig. 2).

LOI due to hypomethylation on the maternal allele results in the loss of function of several genes regulated by the lncRNA KCNQ1OT1 [76], including the tumour suppressor CDKN1C. This loss of CDKN1C is a hallmark Beckwith-Wiedemann overgrowth syndrome, and indeed children with Beckwith-Wiedemann syndrome are at an increased risk of embryonal tumours including RMS [77]. *HRAS*, which is mutated in Costello syndrome, is also located at 11p15.5 and associated with paternal uniparental disomy and predisposition to RMS tumour development [78]. Importantly, the 11p15.5 locus is dysregulated in a high proportion of RMS cases (embryonal and alveolar), even in the setting of no known developmental syndrome [78].

The status of imprinted loci in RMS is not routinely assessed, but this could be accomplished by high

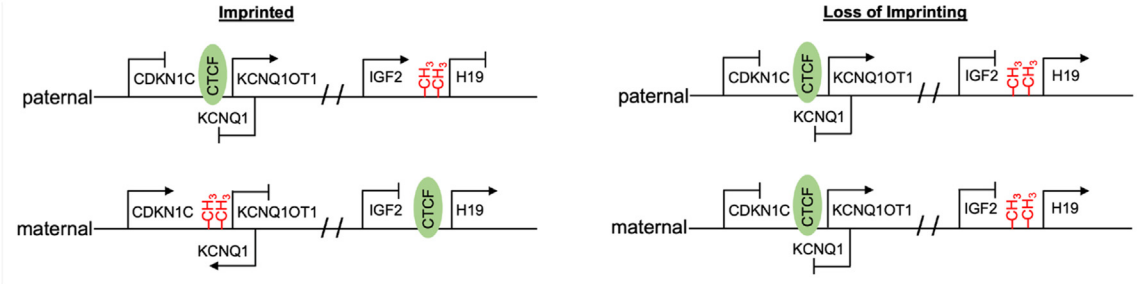

Fig. 2. **Imprinting and loss of imprinting at 11p15.5 locus.** Left: The 11p15.5 locus showing the normal paternal and maternal pattern of methylation (CH3) imprinting. Right: LOI due to hypomethylation of the KCNQ1OT1/CDKN1C locus leads to loss of function of several genes regulated by the lncRNA KCNQ1OT1, including the tumour suppressor CDKN1C. LOI as a result of hypermethylation of the H19/IGF2 locus inhibits H19 expression and results in gain of IGF2 function. Whilst not depicted here, often in RMS these alterations happen together through a paternal uniparental disomy (duplication of entire paternal allele and corresponding loss of maternal). LOI, loss of imprinting; lncRNA, long non-coding RNA.

through-put genomic methods, including assessing methylation status at specific genomic loci and expression of lncRNAs that control these regions. To our knowledge, these assays at imprinted loci have not been used as a prognostic biomarker in RMS (although they are being examined in other cancers). To further understand the role and specific molecular mechanisms regulating LOI in syndromes and RMS tumours may lead to the identification of novel-related drug targets.

2.4. Mutations and amplifications with prognostic impact

The mutational landscape of RMS has been examined using whole-genome sequencing [8,79], whole-exome sequencing [80] and hybrid capture sequencing panels containing selected genes previously shown to be mutated or to have undergone copy number alteration in RMS [81–85]. The frequency of mutations in *PAX3/7-FOXO1*-fusion positive RMS (being primarily driven by the presence of the fusion protein) is consistently found to be lower than in *PAX3/7-FOXO1*-fusion-negative RMS, where approximately 80% of tumours have at least one mutation.

The most common finding in *PAX3/7-FOXO1*-fusion negative tumours was mutated at least one member of the RAS pathway, affecting over half of all cases. Approximately a third of all FN tumours harbour mutations in one of the *RAS* genes (not only predominant *NRAS* but also *HRAS* and *KRAS*). The most recent study described a non-random distribution of these *RAS* mutations with age, with *HRAS* strongly associated with under one-year olds, *KRAS* more frequent in toddlers and *NRAS* showing peak incidence in adolescents [84]. Despite the evidence that *PAX3/7-FOXO1*-fusion negative RMS appears to be RAS driven in the majority of cases, there is no suggestion that somatic RAS pathway gene mutations portend a poorer outcome.

Although more frequent in *PAX3/7-FOXO1*-fusion negative RMS, *TP53* mutations have been found to correlate with outcome in both *PAX3/7-FOXO1*-fusion negative and *PAX3/7-FOXO1*-fusion positive RMS and represents a potentially important biomarker of risk [80,81,84]. As the germline status was not known in some of these studies, it is currently unclear if the worse outcome applies to somatic or germline mutations or both [86]. Introducing a homozygous *tp53* null genotype enhanced invasion and metastasis in a $kRAS^{G12D}$-induced ERMS zebrafish model but did not alter the overall frequency of cancer stem cells suggesting an enhanced pro-metastatic potential association with p53 [87]. *TP53* mutations identified in patients with RMS include insertion and deletions with the loss of heterozygosity or non-synonymous point mutations that confer either a dominant-negative or gain of function effects.

MYOD1 is a member of the basic helix–loop–helix muscle regulatory factor family and is required for muscle differentiation. A particular missense mutation (L122R) has been associated with around a third of the spindle/sclerosing morphologic subtype of RMS, although, importantly, it has also been found in RMS with typical ERMS histology [84] (Fig. 3). This mutation is more frequently seen in tumours from older patients with RMS [84]. The L122R mutation in the *MYOD1* gene uniquely alters the DNA-binding capacity of MYOD1 protein so that it acquires the ability to bind to c-Myc binding sites and enable a Myc-driven transcription program in addition to retaining the ability to bind to MYOD binding sites [88]. $MYOD1^{L122R}$ mutant cases typically show very strong MYOD1 immunohistochemical staining that may be a useful indicator of cases harbouring this somatic $MYOD1^{L122R}$ mutation (Fig. 3).

Although $MYOD1^{L122R}$ account for only ~3% of *PAX3/7-FOXO1*-fusion negative RMS, $MYOD1^{L122R}$ mutated tumours are highly aggressive and have a very poor outcome [84,89,90]. They predominantly, but not exclusively, arise in the head and neck region, and the $MYOD1^{L122R}$ mutation can co-occur with other gene mutations, most notably *PIK3CA*, genes associated with the RAS pathway or *CDKN2A* [84,91]. *CDKN2A* mutations may have prognostic value independent of $MYOD1^{L122R}$, but due to the low overall numbers identified to date, further prospective evaluation is required. $MYOD1^{L122R}$ cases will be excluded from low-risk studies by COG but how to screen for, risk-stratify and treat $MYOD1^{L122R}$ mutant tumours is under consideration in both COG and EpSSG.

Recently, a lack of nuclear MYOD1 immunostaining in ERMS and spindle cell sclerosing RMS, that contrasts to the high level of staining associated with L122R mutations, has also been associated with inferior outcome, although numbers reported are so far small [92]. As MYOD1 is a key determinant of apparent differentiation status in RMS tumours, this lack of nuclear expression may reflect a relatively undifferentiated state.

There are two recurrent amplicons whose prognostic significance has been investigated in *PAX3/7-FOXO1*-fusion positive RMS. The 12q13-14 amplicon containing many genes including *CDK4* has been associated with a poor prognosis in *PAX3/7-FOXO1*-fusion positive RMS [93], although small numbers limited conclusions for CDK4 in a recent study [84]. The 2p24 amplicon containing the *MYCN* oncogene has not consistently been found to be associated with a poor outcome in *PAX3/7-FOXO1*-fusion positive RMS either. Two reports demonstrated a relationship between increased 2p24 copy number and inferior outcome [94,95] although a more recent investigation failed to find a statistically significant association [93]. *CDK4* and *MYCN* amplification will be prospectively assessed for prognostic value independent of PAX3-FOXO1 status in the next COG high-risk RMS study ARST2031 and by EpSSG in the FaR-RMS international trial.

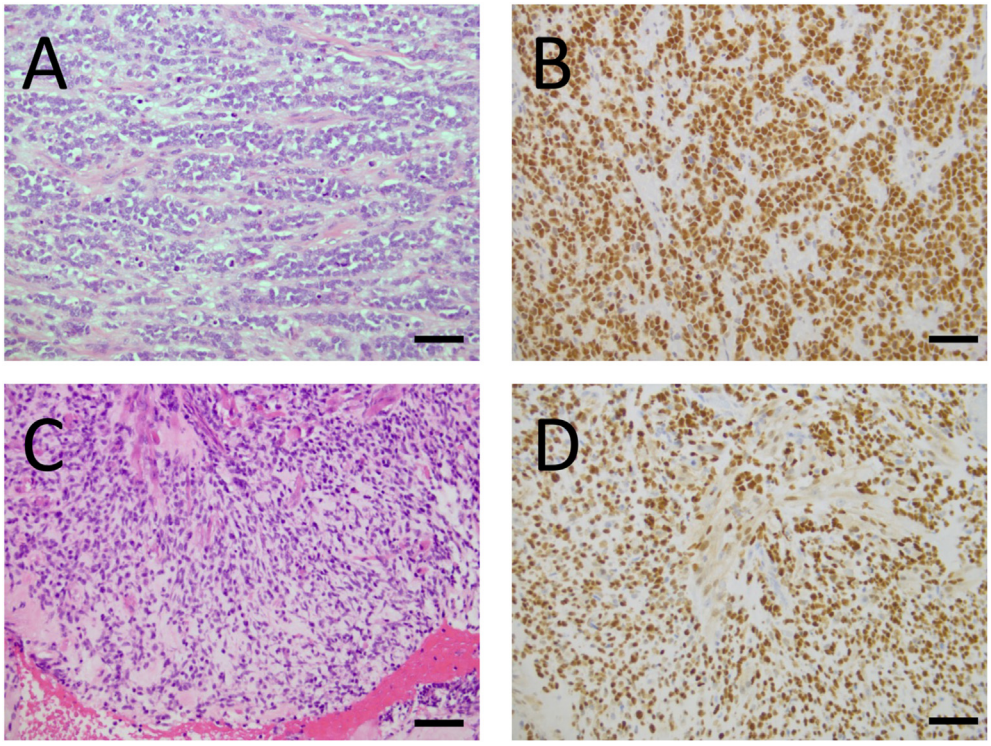

Fig. 3. **Strong MYOD1 immunohistochemistry staining in rhabdomyosarcomas (RMS) with MYOD1 L122R mutation. (a) and (b)** show H&E and MYOD1 immunohistochemistry staining, respectively, of a sclerosing RMS case with mutant in MYOD1. **(c) and (d)** are H&E and MYOD1 immunohistochemistry staining, respectively, of an embryonal RMS with rhabdomyoblastic differentiation (Scale bar, 50 microns).

3. Potential for biomarker detection and analyses using liquid biopsies

Recent technological developments have extended molecular testing of tumours to include the analysis of circulating tumour-derived material shed by tumours into bodily fluids such as blood, urine and saliva. This approach, known as a 'liquid biopsy', may help to overcome the spatial limitations and sampling errors associated with tissue biopsies, as they can capture information from tumour cells which otherwise may not have been sampled due to their anatomical location [87]. Furthermore, as a minimally-invasive technique, liquid biopsies can be collected at multiple time points throughout patient treatment and follow-up. This may not only remove the need for serial tissue biopsies (and thus helps to reduce children's exposure to anaesthesia and imaging procedures) but also may enable the real-time assessment of disease burden and treatment response. As such, liquid biopsies hold enormous potential for RMS screening, diagnosis, risk stratification and monitoring (Fig. 4).

Given that drug resistance and disease recurrence are the major causes of mortality in patients with RMS, there is an urgent need to implement monitoring tools which have the ability to track tumour evolution and detect relapse at the earliest possible stage. Furthermore, the development of a sensitive and specific screening test for those with an increased risk of developing RMS (e.g. children carrying a pathogenic *TP53* germline variant) may help to reduce some of the morbidity associated with late diagnosis. Evidence from studies in adult and other paediatric cancers suggests that liquid biopsies hold promise although the demonstration of clinical utility in RMS is currently limited [91,96–98].

RMS studies to date have primarily focused on the quantification of circulating tumour DNA (ctDNA) in patient blood. Eguchi-Ishimae *et al.* employed qPCR to quantify the levels of the *PAX3-FOXO1*-fusion in serial samples of plasma and bone marrow from a patient with alveolar RMS, demonstrating that ctDNA was detectable prior to radiological evidence of relapse [99]. Similarly, Klega *et al.* used a targeted sequencing panel and whole-genome sequencing to detect *PAX3-FOXO1* rearrangements in ctDNA from 7 patients with alveolar RMS [96]. Importantly, both studies showed that ctDNA levels over time reflected the patient response to treatment, suggesting that ctDNA may be a viable biomarker for monitoring patients with RMS. *PAX3-FOXO1*-fusion transcripts have recently been detected in cell-free exosomal RNA by RT-qPCR [100].

The *PAX3/7-FOXO1*-fusion gene presents an obvious target for analysis given its specificity to RMS and impact upon patient prognosis with potential for high sensitivity and quantitative tracking using droplet digital PCR [11]. However, it is also necessary to identify other

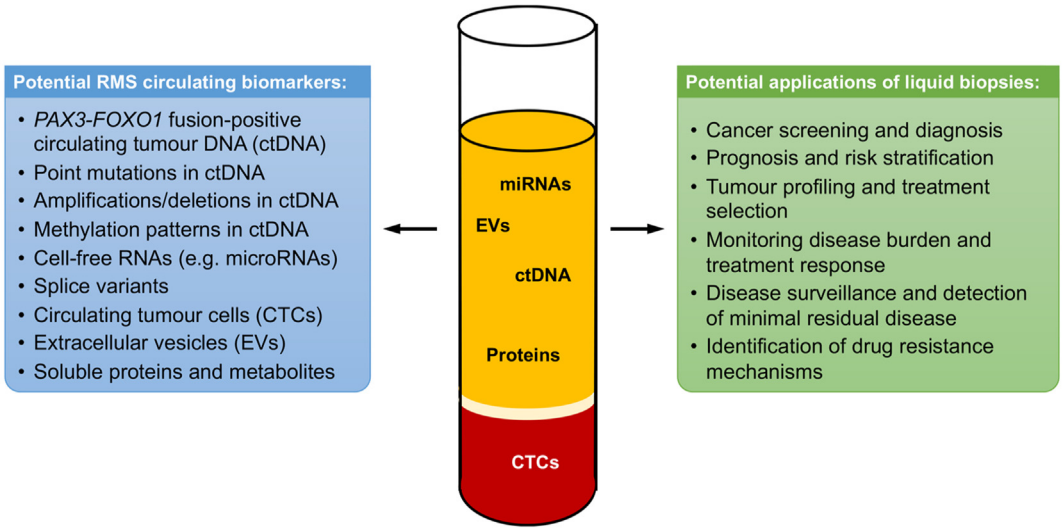

Fig. 4. Overview of liquid biopsy components and potential biomarkers and applications in RMS. CTCs = circulating tumour cells; ctDNA = circulating tumour DNA; EVs = extracellular vesicles; miRNAs = microRNAs.

markers particularly for those patients who are classified as *PAX3/7-FOXO1*-fusion negative. These may include other genomic aberrations described above and use of panel, whole-exome and genome sequencing strategies. Importantly, particularly whole-exome and whole genomic methods will allow the identification of new, clinically-relevant aberrations that may emerge during clonal evolution of the tumour and in relapsed disease which may be of clinical relevance.

The analyses of circulating targets may also include cell-free proteins and RNAs in circulating tumour cells (CTCs) or extracellular vesicles. Some investigations support potential for these other circulating markers for RMS; however, they remain underexplored [101–105]. Future studies may capitalise on new technologies developed for the capture and isolation of CTCs or extracellular vesicles, and may also expand into other biofluids such as urine, saliva or cerebrospinal fluid, which may be particularly relevant for RMS in the bladder, head and neck or brain.

One drawback of liquid biopsies is the potential for variability in sample collection, processing and analysis between centres. It is essential that any liquid biopsy study follows the established preanalytical standards and assay development guidelines (such as those set out by the National Cancer Institute) in order to accurately assess the full potential of liquid biopsies [106]. Further research is also needed into the patient-related factors that may impact biomarker levels, such as the dynamics of ctDNA during patient treatment, as this will help to determine the optimal volumes and time points for collection of liquid biopsy samples. This and the assessment of clinical value will require prospective investigations in the context of international large-scale clinical trials, such as the FaR-RMS and ARST1431 trial, and coordinated collaborative efforts.

4. Prognostic value of gene expression signatures

An alternative strategy using a single genetic mutation or structural aberrations in molecular risk stratification is to harness the prognostic power of gene expression signatures. Identifying 'signatures' of minimal sets of genes whose expression has maximal predictive value are already used clinically (e.g. Mammaprint, BluePrint and Oncotype DX in breast cancer) [107].

Several prognostic signatures have been described for RMS. A signature derived from the ectopic expression of *PAX3-FOXO1,* which was independent of classical clinical risk factors, could stratify ARMS patients into low, intermediate and high-risk categories [108]. This is perhaps unsurprising due to the later finding that *PAX3/7-FOXO1*-fusion negative patients with ARMS generally behaved clinically more like patients with ERMS and had tumour-associated gene expression profiles that were more similar to those in ERMS than fusion positive samples [14]. A subsequent 34 gene signature based on all RMS subtypes [109] was later validated, but correlated with fusion gene status [11]. A signature comprising just five genes demonstrated predictive promise within patients with *PAX3/7-FOXO1*-fusion negative tumours [11] and has also been validated as prognostic for patients within the intermediate risk category established by COG [110].

More recently, the signature called Complexity INdex in SARComas (CINSARC), which was established in adult sarcomas, has been applied to RMS [111]. CINSARC was derived from the differential expression of genes between sarcomas with different clinical grade. CINSARC also incorporated high or low comparative genomic hybridisation genetic imbalances and genes from a previously described chromosome instability signature [112]. CINSARC can successfully predict metastasis in many different sarcoma subtypes with

complex karyotypes and has recently been linked with increased ploidy, intratumour heterogeneity, copy number alterations and a decrease of DNA methylation [113]. Paediatric sarcomas tend to have less chaotic genomes and yet CINSARC correlated with outcome in an RMS cohort that included both fusion gene positive and negative RMS. However, when paediatric and adolescents/young adults were tested separately, only CINSARC in the paediatric cases correlated significantly with outcome [111]. Analyses of additional and larger series are required. The increasing practicality of obtaining and applying gene expression signatures, including the use of formalin fixed samples, enables their validation and potential for future use in risk stratification for RMS.

5. Therapeutic targetability of genetic aberrations

Specific genetic aberrations in RMS tumours, identified through sequencing analyses of tumour samples or liquid biopsies, may indicate targets for therapeutic intervention (Fig. 5).

5.1. Potential to therapeutically target PAX3-FOXO1

PAX3-FOXO1 is an intrinsically disordered protein with no catalytic activity or drug binding pockets which to date has precluded direct pharmacologic targeting. Early efforts in PAX3-FOXO1 biology were directed at inhibiting the downstream transcriptional targets (effectors) of PAX3-FOXO1. However, many studies have shown that the inhibition of even catalytically tractable PAX3-FOXO1 targets, such as receptor tyrosine kinases IGF1R, FGFR4, ALK and MET, did not effectively impair fusion positive RMS cell growth due to rewiring of signalling pathways and the likelihood that their inhibition is not sufficient for full blockade of PAX3-FOXO1 activity [114]. Recent efforts have shifted to targeting proteins that modulate or co-regulate PAX3-FOXO1 activity such as proteins that control the life-cycle of PAX3-FOXO1 (synthesis, activation or degradation of PAX3-FOXO1) [115–118].

Epigenetic modifiers and chromatin remodelers are also relevant although the inhibitors of these generally pose toxicity issues at clinically-relevant doses. These include the HDAC inhibitor entinostat that impairs the growth of PAX3-FOXO1 positive RMS, in part through decreasing PAX3-FOXO1 protein levels [119,120]. Also targeting the bromodomain protein BRD4, that is required for PAX3-FOXO1 activity via its histone acetyltransferase activity, contributes to the decompaction of chromatin and the stability of PAX3-FOXO1 [121].

Efforts to harness immunotherapy techniques to target PAX3-FOXO1 include targeting the PAX3-FOXO1

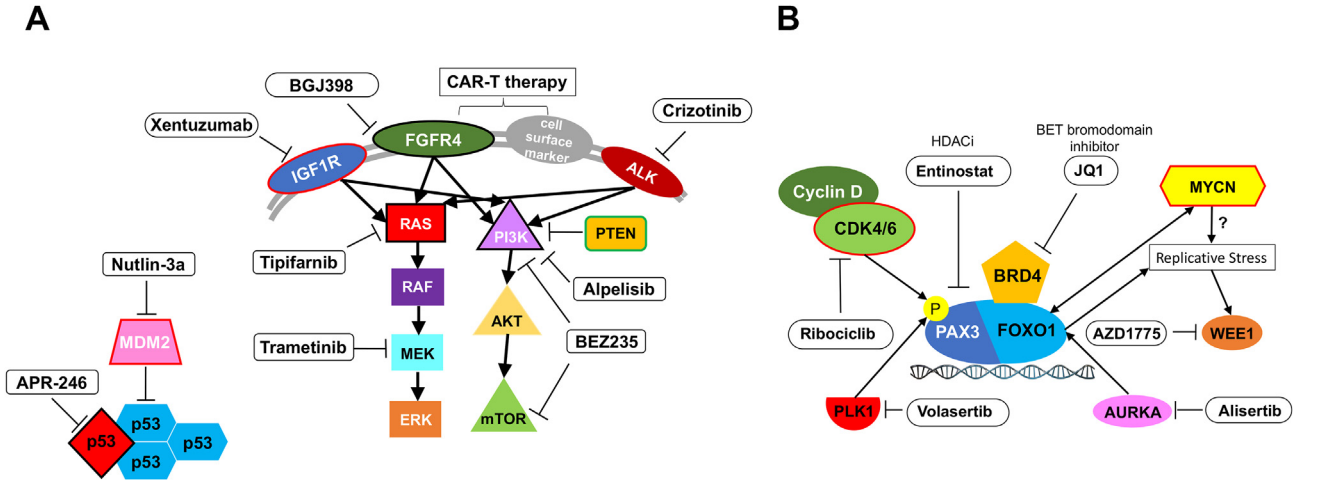

Fig. 5. **Schematic of therapeutic targets in RMS based on somatic genetic aberrations**. (a) Shows potential therapeutic targets in a simplified view of pathways in both *PAX3/7-FOXO1*-fusion gene positive and negative RMS. More than half of fusion negative RMS harbour genetic aberrations activating the RAS pathway, with cross talk to the PI3K pathway, whereas mutation frequency in fusion positive tumours is much less (~12%). However, several RTKs (e.g. FGFRs, IGF1R, ALK and MET) are expressed in both fusion negative and positive RMS – in fusion gene positive cases as a consequence of being downstream transcriptional targets of the fusion protein. Therefore, the hyperactivation of these pathways is common to both subtypes. Similarly, TP53 mutations and MDM2 amplification arise more frequently in fusion negative tumours but are present and potentially targetable in both subtypes. Immune-targeting specific cell surface targets by approaches such as CAR-T therapy also hold some promise. (**b**) Therapeutic targets and agents are indicated involving both direct and indirect targeting of the PAX3-FOXO1-fusion protein. These include targeting binding partners, such as BRD4 and AURKA, or regulators of activity/stability, such as PLK1. In addition, processes induced by the fusion protein, such as replicative stress, create associated vulnerabilities that are targetable (See text for further details). Proteins encoded by genes mutated in RMS are shown with a black outline whereas those amplified or deleted are indicated by a red or green outline, respectively. RMS, rhabdomyosarcoma; RTK, receptor tyrosine kinase.

protein breakpoint epitope via vaccine or targeting cell surface effector proteins of PAX3-FOXO1 (FGFR4, IGF1R and PDGFR alpha) via monoclonal antibodies, Chimeric Antigen Receptor T Cell (CAR-T) or CAR-NK cells [58,122–124]. Targeting myeloid-derived suppressor cells (MDSC) may improve efficacy of CAR therapies in RMS [125]. However, further studies are required to determine the best approach to apply immunotherapy to RMS [123,126].

It is debated whether RMS continue to depend upon PAX3-FOXO1 protein expression for their tumourigenic properties in the recurrent or metastatic clinical setting. At least in primary cell-line derived models, there is some evidence that conditionally-expressed PAX3-FOXO1-driven xenografts can recur even after PAX3-FOXO1 expression is silenced [127]. Demonstrating the loss of the fusion gene in advanced tumour tissue in clinical cohorts would support clonal evolution towards a loss of dependency.

5.2. Putative actionable genetic aberrations

The RAS pathway is altered in the majority of *PAX3/7-FOXO1*-fusion negative RMS, involving *HRAS, KRAS or NRAS* genes in approximately a third of these cases [84]. Candidate compounds in current clinical trials include farnesyl protein transferase inhibitors (FT-ases) such as tipifarnib and lonafarnib, which are designed to prevent the lipidation of RAS proteins, preventing binding to the plasma membrane where RAS activation occurs. The discovery that KRAS and NRAS can undergo lipidation via non-RAS dependant mechanisms, indicates that this class of compounds may be limited to HRAS-mutant-driven tumours [128], which is being assessed in trials that include patients with RMS (NCT04284774 [National Cancer Institute]). More recently, small molecule inhibitors (tetrahydropyridopyrimidines, AMG510 [Amgen Inc.]) have been designed to specifically and irreversibly bind to $KRAS^{G12C}$ and trap it in its inactive GDP-bound state [129,130]. Ongoing clinical trials are assessing these compounds in $KRAS^{G12C}$ mutated tumours and, while these would be expected to help a small minority of patients with RMS, it is hoped that future compounds will target a more expansive array of RAS mutant proteins. The use of mutant-specific inhibitors allows tumour cells to adapt by upregulating signalling through any normal unmutated RAS alleles present. Overcoming this adaptation by using an inhibitor against SHP2 (which mediates signalling from many receptor tyrosine kinase receptors in combination with a mutant -specific RAS inhibitor) proved effective in a preclinical study and suggests that use of vertical pathway inhibition strategies may be necessary to prevent rapid resistance to mutation-specific RAS inhibitors [131].

Beyond the *RAS* genes, there are many mutations in *RAS* pathway genes that contribute to activation of the pathway both above *RAS* with copy number gain or mutation of RTK-encoding genes such as *FGFR4* and less frequently *FGFR1*, *IGF1R*, *PDGFRA*, *MET* and *ALK* as well as mutations in downstream signal transducers such as *BRAF*, all of which can be targeted by approved drugs. However, several trials involving tyrosine kinase inhibitors such as crizotinib (NCT01524926) [132], dasatinib (NCT00464620) [133] and the anti-IGF1R monoclonal antibody R1507 [134] demonstrated little efficacy RMS patients.

Perturbation of the PI3K pathway in RMS is also evident by mutations in *PIK3CA* and loss of PTEN with a prevalence of ~5% and ~1% in *PAX3/7-FOXO1*-fusion negative RMS, respectively [84]. The PI3K pathway is active in the majority of RMS [135] suggesting PI3K/mTOR targeted agents may be broadly effective. Approved drugs specifically targeting *PIK3CA* such as alpelisib are promising in PIK3CA mutated tumours, while broader PI3K/mTOR inhibitors have shown effectiveness in mice in both *FGFR4*-and *PTCH1*-mutant RMS models [136,137].

Defects in the DNA damage response are another major class of genetic aberrations in RMS. *TP53* mutations are the most common in *PAX3/7-FOXO1*-fusion negative RMS (~10–15% of patients) and the small molecule APR-246, which can bind and cause the refolding of mutant *TP53*, is a clinical candidate currently in clinical trials in adult patients [138]. *MDM2* amplification occurs in ~5% of cases [84] indicating that MDM2 antagonists may be of therapeutic value. Adaptation of RMS cells to the transcriptional stress induced by the activity of transcription factors such as the fusion proteins, MYCN and MYOD1 may create dependency on the ATR-CHK1-WEE1 axis. This is supported by the inhibition of WEE1 that has shown potential in a preclinical study [139].

The amplification of *CDK4* occurs in ~10–15% of *PAX3/7-FOXO1*-fusion positive RMS [84,93] with potential prognostic relevance as discussed above. The CDK4/6 inhibitor ribociclib is being investigated in the ESMART trial for paediatric tumours (NCT02813135). Paradoxically, $CDK4^{amp}$ negative patients, that are nonethe-less dependent on CDK4, may benefit more from these inhibitors based on *in vitro* and *in vivo* responses of cell lines with and without *CDK4* amplification [140].

Patients with *MYOD1* mutant tumours are not effectively treated by current protocols and urgently require new tailored treatment options. These may be based on the cooperating mutations in PI3K and RAS pathway genes and/or targeting $MYOD1^{L122R}$ and its role in tumourigenesis; however, directly targeting mutant MYOD1 while leaving the wild-type molecule unaffected would be very challenging.

Finally, the use of proteolysis-targeting chimeras (bifunctional molecules containing two ligands, one to the target molecule to be degraded and one to the E3 ubiquitin ligase) enabling the ubiquitin-proteosome system to degrade any desired molecule is gaining momentum

with more recent advances such as light-activated proteolysis-targeting chimeras allowing spatiotemporal control of their activity to counter potential unwanted side-effects [141]. This will increase therapeutic options for RMS and allow targeting of traditionally undruggable proteins such as transcription factors.

Clinical trial platforms like the 'European Proof-of-Concept Therapeutic Stratification Trial of Molecular Anomalies in Relapsed or Refractory Tumours (ESMART)' (NCT02813135) and the North American NCI pendant of 'Pediatric MATCH treatment trials' (https://childrensoncologygroup.org/index.php/pediatricmatch) in combination with the many sequencing efforts including SMPaeds, MAPPYACTS, INFORM and ITHER enable the evaluation of targeted agents based on molecular profiling data.

The molecular analyses of RMS samples from clinical trial patients in conjunction with establishing patient-derived viable models in centres with relevant expertise will enable determining how representative models are of tumours in patients, both in terms of their molecular make-up and their responses to treatments. Such models, with the inclusion of different cell types that represent the tumour microenvironment, will provide useful tools in identifying and pre-clinically testing novel therapeutic strategies for patients at high-risk.

6. Conclusions and PERSPECTIVE

RMS is a rare cancer with pathological and clinical diversity. Cooperation between national and international cooperative working groups, such as through the INSTRuCT initiative, is advocated to secure clinical data and samples for molecular analyses from sufficient numbers of patients and to integrate data. This will enable robust conclusions to be made for defining clinically useful prognostic and predictive molecular markers as rapidly as possible. Key factors to enable a cooperative approach are the collection of comparable pathological and clinical data and standardisation in the collection times, processing and analyses of samples. This includes use of fresh and snap frozen tumour samples to generate high quality RNAseq data where possible in addition to the use of formalin fixed paraffin embedded material. Encouraging adolescents and young adults, as well as adults, with RMS to register on trials alongside paediatric patients will lead to understanding biological similarities and differences that may be relevant to treatment and management. Based on evidence cited in this review, we propose the following shorter and longer-term priorities for the molecular analyses of samples from patients with RMS enrolled on clinical trials.

6.1. DNA and RNA analyses of RMS tumour samples

Whilst PAX3-FOXO1 status is accepted as poor prognostic indicator, it is grouped together with PAX7-FOXO1 in current protocols for risk stratification. However, the prognosis of patients with PAX7-FOXO1 tumours has been indicated better than those with PAX3-FOXO1, although not in all studies and the numbers of cases reported in each study is small. Therefore, prospective assessment in a multivariate approach is crucial in addition to assessing the impact of using fusion gene status for risk stratification in current trials. Although challenging to draw firm conclusions, data for rarer fusions involving PAX or FOXO1 genes should be accrued for future assessment. This includes the evaluation of recurrent *NCOA2* or *VGLL2* gene fusions found in patients with MYOD1 mutant-negative spindle cell RMS where published observations are not sufficiently clear to inform decisions for patients.

We also propose DNA sequencing to include high priority genes (including but not limited to *MYOD1, TP53*, *CDKN2A, CDK4* and *MYCN*). The poor survival rates associated with *MYOD1* L122R mutations, which is not limited to the spindle/sclerosing pathology, is either being used or considered for escalating treatment intensity. Ultimately, new treatments options need to be identified for patients with *MYOD1* mutations, and other genetic aberrations that are validated as high-risk, where current treatments fail. Extending DNA sequencing to larger panels of genes, exome or whole-genome wherever possible is highly desirable to enable the validation and identification of prognostic aberrations, including potential cooperating events.

Similarly, the RNA sequencing of tumour samples at baseline will also allow the further evaluation of the prognostic value of sequence variants in RMS-relevant genes and the definition of variant and new gene fusions. Furthermore, RNA-sequencing, or analyses of specific expressed prognostic gene signatures (5-gene RMS signature and CINSARC), will enable the further evaluation of these previously defined gene signatures and the potential to identify and assess new prognostic gene expression signatures. RNA sequencing, including single cells, of RMS samples at relapse together with upfront samples in the context of clinical trials, will facilitate understanding the molecular biology of RMS tumour progression, increasing age-related poorer outcomes and the development of new treatment strategies.

6.2. Germline assessment

A clinical diagnosis of Li Fraumeni syndrome or neurofibromatosis type 1 should be considered, especially in younger patients diagnosed with RMS that are PAX3/7-FOXO1-negative. Tumour and germline genetic assessment should ideally be conducted in parallel. We also encourage contributing to explorative studies to identify new RMS predisposition genes, including possible germline genes that may predispose to fusion gene formation. Correlating P/LP germline variant data with tumour characteristics and disease course may

inform treatment and intensity options that minimise toxicities and risk of secondary malignancies.

6.3. Liquid biopsies

Liquid biopsies hold enormous potential for detecting clinically-relevant molecular aberrations for diagnosis/prognosis, assessing response to treatments over time and early detection and characterization of relapses. Sampling is less invasive than tissue biopsies, which makes collecting material over a time-course possible. Evaluating the timing of collection of samples and proper assay and data analyses needed to perform to bring most benefit to patients with RMS is a priority to determine in the context of RMS clinical trials.

Quantitative measures of response to therapy in liquid biopsies using tumour-specific molecular markers may ultimately supplement predicting responses to treatment and potentially replace the need to take biopsies. Proof-of-principle studies should be a priority to assess *PAX3/7-FOXO1* and tumour-specific mutations based on previous feasibility studies. Quantitative tracking of ctDNA presence/absence and correlation with event-free survival and radiological/metabolic measures of response in patients hold promise. More extensive analyses of ctDNA, using panel sequencing or whole-genome or -exome sequencing, has potential to capture emerging genetic alterations that may be clinically-relevant by identifying new treatment options at relapse. In addition, the value of assessing ctDNA-specific methylation patterns is anticipated to enhance sensitivity. Sample collections will enable exploring the value of assessing nucleic acids and proteins in CTCs and exosomes which, in contrast to ctDNA, represent a viable component of tumours. Robust collection protocols for plasma/buffy coat and potentially other liquid biopsies, with harmonisation of collection of time points across cooperative group studies will accelerate progress.

6.4. Patient-derived models

Where laboratories have the necessary expertise and resources, *in vitro* and *in vivo* models of biologically distinct subtypes of RMS at diagnosis and relapse will be derived that can support efforts in the research community to further develop new treatments. The molecular characterisation of models and comparison with data from samples from which they were derived as well as co-clinical testing of responses to treatment in patients and their parallel models will indicate the representativeness of newly derived models. Studies including such models will identify new treatment strategies, establish molecular characteristics that are predictive of response, validate biomarkers to reflect target engagement and determine molecular mechanisms of resistance and possible ways to overcome this.

6.5. Data integration

The integration of clinical, imaging and pathological characteristics of disease with molecular markers will be useful for patient care and important for current and future research. Molecular data such as the presence or absence of gene fusions, sequence variants in genes or gene expression signatures and quantitative measures of response to treatment, as evidenced by changes in ctDNA markers and imaging features will be essential to generate and compile. Better ways to assess the risk of failure may emerge from multivariate analyses of new risk stratification algorithms incorporating molecular and clinical features. The INSTRuCT rhabdomyosarcoma data commons, part of the Pediatric Cancer Data Commons at University of Chicago (http://commons.cri.uchicago.edu), houses harmonised clinical data from completed clinical trials and includes information on almost 5000 children. Additional data from studies is being added, from both children and young adults. Where possible, data are being linked to other sources of information, such as genomic data and biospecimen availability. This growing set of rich data has the potential to be harnessed for the development of better risk stratification using machine learning and other quantitative methods along with prospectively collected data.

Together, this approach will create collections of molecularly characterised samples and models with corresponding pathological, clinical and imaging data that will support ongoing and future research and translational efforts within and across cooperative groups that aim to improve the outcomes of young patients with RMS.

Author contribution statement

Re: Molecular testing of rhabdomyosarcoma in clinical trials to improve risk stratification and outcome: A consensus view from EpSSG (European paediatric Soft Tissue Sarcoma Study Group), COG (Children's Oncology Group) and CWS (Cooperative Weichteilsarkom-Studiengruppe)

Concept for the review: Simone Hettmer, Corinne Linardic, Anna Kelsey, Erin R. Rudzinski, Christian Vokuhl and Janet Shipley, Original drafting of sections: Simone Hettmer, Corinne Linardic, Joanna Selfe, Olivia Ruhen, Alexander R. Kovach, Janet Shipley, Contributions to figures and the table: Simone Hettmer, Corinne Linardic, Anna Kelsey, Olivia Ruhen, Alexander R. Kovach, Philip J. Lupo, Javed Khan, Janet Shipley Contributed to the consensus view of the working groups: Ewa Koscielniak, Douglas S. Hawkins, Gianni Bisogno, Monika Sparber-Sauer, Rajkumar Venkatramani, Johannes H.M. Merks, Writing, reviewing and editing: All authors.

Funding

This review was supported by the Chris Lucas Trust and Alice's Arc, Children's Cancer Charity. The International Soft Tissue Sarcoma Consortium and the Pediatric Cancer Data Commons are supported in part by Cancer Research Foundation, Children's Research Foundation, Comer Development Board, KickCancer, King Baudouin Foundation, Rally Foundation for Childhood Cancer Research, Seattle Children's Foundation from Kat's Crew Guild through the Sarcoma Research Fund, St Baldrick's Foundation, and The Andrew McDonough B + Foundation.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Acknowledgements

The authors thank Suzi Birz for her tireless support through INSTRuCT in organising meetings and providing information that facilitated this review. This work was made possible through the efforts of Children's Oncology Group, Cooperative Weichteilsarkom Studiengruppe der GPOH (CWS), The European paediatric Soft tissue sarcoma Study Group, MMT Malignant Mesenchymal Tumour Committee, STSC AIEOP Italian Soft Tissue Sarcoma Committee.

References

[1] Sultan I, Qaddoumi I, Yaser S, Rodriguez-Galindo C, Ferrari A. Comparing adult and pediatric rhabdomyosarcoma in the surveillance, epidemiology and end results program, 1973 to 2005: an analysis of 2,600 patients. J Clin Oncol 2009;27(20):3391–7.

[2] Chisholm JC, Marandet J, Rey A, Scopinaro M, de Toledo JS, Merks JH, O'Meara A, Stevens MC, Oberlin O. Prognostic factors after relapse in nonmetastatic rhabdomyosarcoma: a nomogram to better define patients who can be salvaged with further therapy. J Clin Oncol 2011;29(10):1319–25.

[3] Oberlin O, Rey A, Lyden E, Bisogno G, Stevens MC, Meyer WH, Carli M, Anderson JR. Prognostic factors in metastatic rhabdomyosarcomas: results of a pooled analysis from United States and European cooperative groups. J Clin Oncol 2008;26(14):2384–9.

[4] Rudzinski ER, Anderson JR, Hawkins DS, Skapek SX, Parham DM, Teot LA. The World Health organization classification of skeletal muscle tumors in pediatric rhabdomyosarcoma: a report from the children's oncology group. Arch Pathol Lab Med 2015;139(10):1281–7.

[5] Barr FG, Galili N, Holick J, Biegel JA, Rovera G, Emanuel BS. Rearrangement of the PAX3 paired box gene in the paediatric solid tumour alveolar rhabdomyosarcoma. Nat Genet 1993;3(2):113–7.

[6] Davis RJ, D'Cruz CM, Lovell MA, Biegel JA, Barr FG. Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. Cancer Res 1994;54(11):2869–72.

[7] Galili N, Davis RJ, Fredericks WJ, Mukhopadhyay S, Rauscher 3rd FJ, Emanuel BS, Rovera G, Barr FG. Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat Genet 1993;5(3):230–5.

[8] Shern JF, Chen L, Chmielecki J, Wei JS, Patidar R, Rosenberg M, Ambrogio L, Auclair D, Wang J, Song YK, Tolman C, Hurd L, Liao H, Zhang S, Bogen D, Brohl AS, Sindiri S, Catchpoole D, Badgett T, Getz G, Mora J, Anderson JR, Skapek SX, Barr FG, Meyerson M, Hawkins DS, Khan J. Comprehensive genomic analysis of rhabdomyosarcoma reveals a landscape of alterations affecting a common genetic axis in fusion-positive and fusion-negative tumors. Cancer Discov 2014;4(2):216–31.

[9] Weber-Hall S, Anderson J, McManus A, Abe S, Nojima T, Pinkerton R, Pritchard-Jones K, Shipley J. Gains, losses, and amplification of genomic material in rhabdomyosarcoma analyzed by comparative genomic hybridization. Cancer Res 1996;56(14):3220–4.

[10] Davicioni E, Anderson MJ, Finckenstein FG, Lynch JC, Qualman SJ, Shimada H, Schofield DE, Buckley JD, Meyer WH, Sorensen PH, Triche TJ. Molecular classification of rhabdomyosarcoma–genotypic and phenotypic determinants of diagnosis: a report from the Children's Oncology Group. Am J Pathol 2009;174(2):550–64.

[11] Missiaglia E, Williamson D, Chisholm J, Wirapati P, Pierron G, Petel F, Concordet JP, Thway K, Oberlin O, Pritchard-Jones K, Delattre O, Delorenzi M, Shipley J. PAX3/FOXO1 fusion gene status is the key prognostic molecular marker in rhabdomyosarcoma and significantly improves current risk stratification. J Clin Oncol 2012;30(14):1670–7.

[12] Selfe J, Olmos D, Al-Saadi R, Thway K, Chisholm J, Kelsey A, Shipley J. Impact of fusion gene status versus histology on risk-stratification for rhabdomyosarcoma: retrospective analyses of patients on UK trials. Pediatr Blood Cancer 2017;64(7).

[13] Skapek SX, Anderson J, Barr FG, Bridge JA, Gastier-Foster JM, Parham DM, Rudzinski ER, Triche T, Hawkins DS. PAX-FOXO1 fusion status drives unfavorable outcome for children with rhabdomyosarcoma: a children's oncology group report. Pediatr Blood Cancer 2013;60(9):1411–7.

[14] Williamson D, Missiaglia E, de Reynies A, Pierron G, Thuille B, Palenzuela G, Thway K, Orbach D, Lae M, Freneaux P, Pritchard-Jones K, Oberlin O, Shipley J, Delattre O. Fusion gene-negative alveolar rhabdomyosarcoma is clinically and molecularly indistinguishable from embryonal rhabdomyosarcoma. J Clin Oncol 2010;28(13):2151–8.

[15] Hawkins DS, Bisogno G, Koscielniak E. Introducing INSTRuCT: an international effort to promote cooperation and data sharing. Pediatr Blood Cancer; 2020. p. e28701.

[16] Rudzinski ER, Kelsey A, Vokuhl C, Linardic CM, Shipley J, Hettmer S, Koscielniak E, Hawkins DS, Bisogno G. Pathology of childhood rhabdomyosarcoma: a consensus opinion document from the children's oncology group, European paediatric soft tissue sarcoma study group, and the cooperative Weichteilsarkom Studiengruppe. Pediatr Blood Cancer 2021;68(3):e28798.

[17] Arnold MA, Anderson JR, Gastier-Foster JM, Barr FG, Skapek SX, Hawkins DS, Raney Jr RB, Parham DM, Teot LA, Rudzinski ER, Walterhouse DO. Histology, fusion status, and outcome in alveolar rhabdomyosarcoma with low-risk clinical features: a report from the children's oncology group. Pediatr Blood Cancer 2016;63(4):634–9.

[18] Hibbitts E, Chi YY, Hawkins DS, Barr FG, Bradley JA, Dasgupta R, Meyer WH, Rodeberg DA, Rudzinski ER, Spunt SL, Skapek SX, Wolden SL, Arndt CAS. Refinement of risk stratification for childhood rhabdomyosarcoma using FOXO1 fusion status in addition to established clinical outcome

predictors: a report from the Children's Oncology Group. Cancer Med 2019;8(14):6437–48.
[19] Kazanowska B, Reich A, Stegmaier S, Bekassy AN, Leuschner I, Chybicka A, Koscielniak E. Pax3-fkhr and pax7-fkhr fusion genes impact outcome of alveolar rhabdomyosarcoma in children. Fetal Pediatr Pathol 2007;26(1):17–31.
[20] Sorensen PH, Lynch JC, Qualman SJ, Tirabosco R, Lim JF, Maurer HM, Bridge JA, Crist WM, Triche TJ, Barr FG. PAX3-FKHR and PAX7-FKHR gene fusions are prognostic indicators in alveolar rhabdomyosarcoma: a report from the children's oncology group. J Clin Oncol 2002;20(11):2672–9.
[21] Stegmaier S, Bielack SS, Leuschner I, Klingebiel T, Koscielniak E. Questionable universal validity of PAX3/FOXO1 fusion gene status as molecular marker for improvement of risk stratification in rhabdomyosarcoma therapy. J Clin Oncol 2012; 30(32):4039–40. author reply 4040-1.
[22] Stegmaier S, Poremba C, Schaefer KL, Leuschner I, Kazanowska B, Bekassy AN, Bielack SS, Klingebiel T, Koscielniak E. Prognostic value of PAX-FKHR fusion status in alveolar rhabdomyosarcoma: a report from the cooperative soft tissue sarcoma study group (CWS). Pediatr Blood Cancer 2011; 57(3):406–14.
[23] Heske CM, Chi YY, Venkatramani R, Li M, Arnold MA, Dasgupta R, Hiniker SM, Hawkins DS, Mascarenhas L. Survival outcomes of patients with localized FOXO1 fusion-positive rhabdomyosarcoma treated on recent clinical trials: a report from the Soft Tissue Sarcoma Committee of the Children's Oncology Group. Cancer 2021;127(6):946–56.
[24] Azorsa DO, Bode PK, Wachtel M, Cheuk ATC, Meltzer PS, Vokuhl C, Camenisch U, Khov HL, Bode B, Schafer BW, Khan J. Immunohistochemical detection of PAX-FOXO1 fusion proteins in alveolar rhabdomyosarcoma using breakpoint specific monoclonal antibodies. Mod Pathol 2021;34(4):748–57.
[25] Rudzinski ER, Anderson JR, Lyden ER, Bridge JA, Barr FG, Gastier-Foster JM, Bachmeyer K, Skapek SX, Hawkins DS, Teot LA, Parham DM, Myogenin AP2beta. NOS-1, and HMGA2 are surrogate markers of fusion status in rhabdomyosarcoma: a report from the soft tissue sarcoma committee of the children's oncology group. Am J Surg Pathol 2014;38(5): 654–9.
[26] Wachtel M, Runge T, Leuschner I, Stegmaier S, Koscielniak E, Treuner J, Odermatt B, Behnke S, Niggli FK, Schafer BW. Subtype and prognostic classification of rhabdomyosarcoma by immunohistochemistry. J Clin Oncol 2006; 24(5):816–22.
[27] Barr FG, Qualman SJ, Macris MH, Melnyk N, Lawlor ER, Strzelecki DM, Triche TJ, Bridge JA, Sorensen PH. Genetic heterogeneity in the alveolar rhabdomyosarcoma subset without typical gene fusions. Cancer Res 2002;62(16):4704–10.
[28] Sumegi J, Streblow R, Frayer RW, Dal Cin P, Rosenberg A, Meloni-Ehrig A, Bridge JA. Recurrent t(2;2) and t(2;8) translocations in rhabdomyosarcoma without the canonical PAX-FOXO1 fuse PAX3 to members of the nuclear receptor transcriptional coactivator family. Genes Chromosomes Cancer 2010;49(3):224–36.
[29] Wachtel M, Dettling M, Koscielniak E, Stegmaier S, Treuner J, Simon-Klingenstein K, Buhlmann P, Niggli FK, Schafer BW. Gene expression signatures identify rhabdomyosarcoma subtypes and detect a novel t(2;2)(q35;p23) translocation fusing PAX3 to NCOA1. Cancer Res 2004;64(16):5539–45.
[30] Alaggio R, Zhang L, Sung YS, Huang SC, Chen CL, Bisogno G, Zin A, Agaram NP, LaQuaglia MP, Wexler LH, Antonescu CR. A molecular study of pediatric spindle and sclerosing rhabdomyosarcoma: identification of novel and recurrent VGLL2-related fusions in infantile cases. Am J Surg Pathol 2016;40(2): 224–35.
[31] Mosquera JM, Sboner A, Zhang L, Kitabayashi N, Chen CL, Sung YS, Wexler LH, LaQuaglia MP, Edelman M, Sreekantaiah C, Rubin MA, Antonescu CR. Recurrent NCOA2 gene rearrangements in congenital/infantile spindle cell rhabdomyosarcoma. Genes Chromosomes Cancer 2013;52(6):538–50.
[32] Butel T, Karanian M, Pierron G, Orbach D, Ranchere D, Cozic N, Galmiche L, Coulomb A, Corradini N, Lacour B, Proust S, Guerin F, Boutroux H, Rome A, Mansuy L, Verite C, Defachelles AS, Tirode F, Minard-Colin V. Integrative clinical and biopathology analyses to understand the clinical heterogeneity of infantile rhabdomyosarcoma: a report from the French MMT committee. Cancer Med 2020;9(8):2698–709.
[33] Whittle SB, Hicks MJ, Roy A, Vasudevan SA, Reddy K, Venkatramani R. Congenital spindle cell rhabdomyosarcoma. Pediatr Blood Cancer 2019;66(11):e27935.
[34] Cyrta J, Gauthier A, Karanian M, Vieira AF, Cardoen L, Jehanno N, Bouvet M, Bouvier C, Komuta M, Le Loarer F, Orbach D, Rome A, Minard-Colin V, Brichard B, Pluchart C, Thebaud E, Renard M, Pannier S, Brisse H, Petit P, Benoist C, Schleiermacher G, Geoerger B, Vincent-Salomon A, Freneaux P, Pierron G. Infantile rhabdomyosarcomas with VGLL2 rearrangement are not always an indolent disease: a study of 4 aggressive cases with clinical, pathologic, molecular, and radiologic findings. Am J Surg Pathol 2021; 45(6):854–67.
[35] Karanian M, Pissaloux D, Gomez-Brouchet A, Chevenet C, Le Loarer F, Fernandez C, Minard V, Corradini N, Castex MP, Duc-Gallet A, Blay JY, Tirode F. SRF-FOXO1 and SRF-NCOA1 fusion genes delineate a distinctive subset of well-differentiated rhabdomyosarcoma. Am J Surg Pathol 2020; 44(5):607–16.
[36] Agaram NP, Zhang L, Sung YS, Cavalcanti MS, Torrence D, Wexler L, Francis G, Sommerville S, Swanson D, Dickson BC, Suurmeijer AJH, Williamson R, Antonescu CR. Expanding the spectrum of intraosseous rhabdomyosarcoma: correlation between 2 distinct gene fusions and phenotype. Am J Surg Pathol 2019;43(5):695–702.
[37] Dashti NK, Wehrs RN, Thomas BC, Nair A, Davila J, Buckner JC, Martinez AP, Sukov WR, Halling KC, Howe BM, Folpe AL. Spindle cell rhabdomyosarcoma of bone with FUS-TFCP2 fusion: confirmation of a very recently described rhabdomyosarcoma subtype. Histopathology 2018;73(3):514–20.
[38] Watson S, Perrin V, Guillemot D, Reynaud S, Coindre JM, Karanian M, Guinebretiere JM, Freneaux P, Le Loarer F, Bouvet M, Galmiche-Rolland L, Larousserie F, Longchampt E, Ranchere-Vince D, Pierron G, Delattre O, Tirode F. Transcriptomic definition of molecular subgroups of small round cell sarcomas. J Pathol 2018;245(1):29–40.
[39] Kallen ME, Hornick JL. From the ashes of "Ewing-like" sarcoma: a contemporary update of the classification, immunohistochemistry, and molecular genetics of round cell sarcomas. Semin Diagn Pathol 2022;39(1):29–37.
[40] Montoya-Cerrillo DM, Diaz-Perez JA, Velez-Torres JM, Montgomery EA, Rosenberg AE. Novel fusion genes in spindle cell rhabdomyosarcoma: the spectrum broadens. Genes Chromosomes Cancer 2021;60(10):687–94.
[41] Ognjanovic S, Olivier M, Bergemann TL, Hainaut P. Sarcomas in TP53 germline mutation carriers: a review of the IARC TP53 database. Cancer 2012;118(5):1387–96.
[42] Sung L, Anderson JR, Arndt C, Raney RB, Meyer WH, Pappo AS. Neurofibromatosis in children with rhabdomyosarcoma: a report from the intergroup rhabdomyosarcoma study IV. J Pediatr 2004;144(5):666–8.
[43] Hettmer S, Teot LA, Kozakewich H, Werger AM, Davies KJ, Fletcher CD, Grier HE, Rodriguez-Galindo C, Wagers AJ. Myogenic tumors in nevoid Basal cell carcinoma syndrome. J Pediatr Hematol Oncol 2015;37(2):147–9.
[44] Stewart DR, Best AF, Williams GM, Harney LA, Carr AG, Harris AK, Kratz CP, Dehner LP, Messinger YH, Rosenberg PS, Hill DA, Schultz KAP. Neoplasm risk among

individuals with a pathogenic germline variant in DICER1. J Clin Oncol 2019;37(8):668–76.
[45] Gripp KW. Tumor predisposition in Costello syndrome. Am J Med Genet C Semin Med Genet 2005;137C(1):72–7.
[46] Kratz CP, Franke L, Peters H, Kohlschmidt N, Kazmierczak B, Finckh U, Bier A, Eichhorn B, Blank C, Kraus C, Kohlhase J, Pauli S, Wildhardt G, Kutsche K, Auber B, Christmann A, Bachmann N, Mitter D, Cremer FW, Mayer K, Daumer-Haas C, Nevinny-Stickel-Hinzpeter C, Oeffner F, Schluter G, Gencik M, Uberlacker B, Lissewski C, Schanze I, Greene MH, Spix C, Zenker M. Cancer spectrum and frequency among children with Noonan, Costello, and cardio-facio-cutaneous syndromes. Br J Cancer 2015;112(8):1392–7.
[47] Kuroiwa M, Sakamoto J, Shimada A, Suzuki N, Hirato J, Park MJ, Sotomatsu M, Hayashi Y. Manifestation of alveolar rhabdomyosarcoma as primary cutaneous lesions in a neonate with Beckwith-Wiedemann syndrome. J Pediatr Surg 2009;44(3):e31–5.
[48] Piersigilli F, Auriti C, Mondi V, Francalanci P, Salvatori G, Danhaive O. Decreased CDKN1C expression in congenital alveolar rhabdomyosarcoma associated with beckwith-wiedemann syndrome. Indian J Pediatr 2016;83(12–13):1476–8.
[49] Smith AC, Squire JA, Thorner P, Zielenska M, Shuman C, Grant R, Chitayat D, Nishikawa JL, Weksberg R. Association of alveolar rhabdomyosarcoma with the Beckwith-Wiedemann syndrome. Pediatr Dev Pathol 2001;4(6):550–8.
[50] Walsh MF, Chang VY, Kohlmann WK, Scott HS, Cunniff C, Bourdeaut F, Molenaar JJ, Porter CC, Sandlund JT, Plon SE, Wang LL, Savage SA. Recommendations for childhood cancer screening and surveillance in DNA repair disorders. Clin Cancer Res 2017;23(11):e23–31.
[51] Miller RW, Rubinstein JH. Tumors in rubinstein-taybi syndrome. Am J Med Genet 1995;56(1):112–5.
[52] Akhavanfard S, Padmanabhan R, Yehia L, Cheng F, Eng C. Comprehensive germline genomic profiles of children, adolescents and young adults with solid tumors. Nat Commun 2020;11(1):2206.
[53] Grobner SN, et al. The landscape of genomic alterations across childhood cancers. Nature 2018;555(7696):321–7.
[54] Kim J, Light N, Subasri V, Young EL, Wegman-Ostrosky T, Barkauskas DA, Hall D, Lupo PJ, Patidar R, Maese LD, Jones K, Wang M, Tavtigian SV, Wu D, Shlien A, Telfer F, Goldenberg A, Skapek SX, Wei JS, Wen X, Catchpoole D, Hawkins DS, Schiffman JD, Khan J, Malkin D, Stewart DR. Pathogenic germline variants in cancer susceptibility genes in children and young adults with rhabdomyosarcoma. JCO Precis Oncol 2021;5.
[55] Li H, Sisoudiya SD, Martin-Giacalone BA, Khayat MM, Dugan-Perez S, Marquez-Do DA, Scheurer ME, Muzny D, Boerwinkle E, Gibbs RA, Chi YY, Barkauskas DA, Lo T, Hall D, Stewart DR, Schiffman JD, Skapek SX, Hawkins DS, Plon SE, Sabo A, Lupo PJ. Germline cancer predisposition variants in pediatric rhabdomyosarcoma: a report from the children's oncology group. J Natl Cancer Inst 2021;113(7):875–83.
[56] Zhang J, Walsh MF, Wu G, Edmonson MN, Gruber TA, Easton J, Hedges D, Ma X, Zhou X, Yergeau DA, Wilkinson MR, Vadodaria B, Chen X, McGee RB, Hines-Dowell S, Nuccio R, Quinn E, Shurtleff SA, Rusch M, Patel A, Becksfort JB, Wang S, Weaver MS, Ding L, Mardis ER, Wilson RK, Gajjar A, Ellison DW, Pappo AS, Pui CH, Nichols KE, Downing JR. Germline mutations in predisposition genes in pediatric cancer. N Engl J Med 2015;373(24):2336–46.
[57] Ballinger ML, Thomas DM. International sarcoma Kindred S., sarcoma and germ-line DICER1 mutations - authors' reply. Lancet Oncol 2016;17(11):e471.
[58] Huang X, Park H, Greene J, Pao J, Mulvey E, Zhou SX, Albert CM, Moy F, Sachdev D, Yee D, Rader C, Hamby CV, Loeb DM, Cairo MS, Zhou X. IGF1R- and ROR1-specific CAR T cells as a potential therapy for high risk sarcomas. PLoS One 2015;10(7):e0133152.
[59] Mody RJ, Wu YM, Lonigro RJ, Cao X, Roychowdhury S, Vats P, Frank KM, Prensner JR, Asangani I, Palanisamy N, Dillman JR, Rabah RM, Kunju LP, Everett J, Raymond VM, Ning Y, Su F, Wang R, Stoffel EM, Innis JW, Roberts JS, Robertson PL, Yanik G, Chamdin A, Connelly JA, Choi S, Harris AC, Kitko C, Rao RJ, Levine JE, Castle VP, Hutchinson RJ, Talpaz M, Robinson DR, Chinnaiyan AM. Integrative clinical sequencing in the management of refractory or relapsed cancer in youth. JAMA 2015;314(9):913–25.
[60] Diller L, Sexsmith E, Gottlieb A, Li FP, Malkin D. Germline p53 mutations are frequently detected in young children with rhabdomyosarcoma. J Clin Invest 1995;95(4):1606–11.
[61] Archer NM, Amorim RP, Naves R, Hettmer S, Diller LR, Ribeiro KB, Rodriguez-Galindo C. An increased risk of second malignant neoplasms after rhabdomyosarcoma: population-based evidence for a cancer predisposition syndrome? Pediatr Blood Cancer 2016;63(2):196–201.
[62] Lupo PJ, Danysh HE, Plon SE, Curtin K, Malkin D, Hettmer S, Hawkins DS, Skapek SX, Spector LG, Papworth K, Melin B, Erhardt EB, Grufferman S, Schiffman JD. Family history of cancer and childhood rhabdomyosarcoma: a report from the Children's Oncology Group and the Utah Population Database. Cancer Med 2015;4(5):781–90.
[63] Hettmer S, Archer NM, Somers GR, Novokmet A, Wagers AJ, Diller L, Rodriguez-Galindo C, Teot LA, Malkin D. Anaplastic rhabdomyosarcoma in TP53 germline mutation carriers. Cancer 2014;120(7):1068–75.
[64] Crucis A, Richer W, Brugieres L, Bergeron C, Marie-Cardine A, Stephan JL, Girard P, Corradini N, Munzer M, Lacour B, Minard-Colin V, Sarnacki S, Ranchere-Vince D, Orbach D, Bourdeaut F. Rhabdomyosarcomas in children with neurofibromatosis type I: a national historical cohort. Pediatr Blood Cancer 2015;62(10):1733–8.
[65] Ferrari A, Bisogno G, Macaluso A, Casanova M, D'Angelo P, Pierani P, Zanetti I, Alaggio R, Cecchetto G, Carli M. Soft-tissue sarcomas in children and adolescents with neurofibromatosis type 1. Cancer 2007;109(7):1406–12.
[66] Pondrom M, Bougeard G, Karanian M, Bonneau-Lagacherie J, Boulanger C, Boutroux H, Briandet C, Chevreau C, Corradini N, Coze C, Defachelles AS, Galmiche-Roland L, Orbach D, Piguet C, Scoazec JY, Verite C, Willems M, Frebourg T, Minard V, Brugieres L. Rhabdomyosarcoma associated with germline TP53 alteration in children and adolescents: the French experience. Pediatr Blood Cancer 2020;67(9):e28486.
[67] MacDonald WA, Mann MRW. Long noncoding RNA functionality in imprinted domain regulation. PLoS Genet 2020;16(8):e1008930.
[68] Tucci V, Isles AR, Kelsey G, Ferguson-Smith AC, Erice Imprinting G. Genomic imprinting and physiological processes in mammals. Cell 2019;176(5):952–65.
[69] Hanna CW, Kelsey G. The specification of imprints in mammals. Heredity (Edinb) 2014;113(2):176–83.
[70] Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. J Mol Biol 1987;196(2):261–82.
[71] Kim S, Yu NK, Kaang BK. CTCF as a multifunctional protein in genome regulation and gene expression. Exp Mol Med 2015;47:e166.
[72] Scelfo RA, Schwienbacher C, Veronese A, Gramantieri L, Bolondi L, Querzoli P, Nenci I, Calin GA, Angioni A, Barbanti-Brodano G, Negrini M. Loss of methylation at chromosome 11p15.5 is common in human adult tumors. Oncogene 2002;21(16):2564–72.
[73] Bliek J, Terhal P, van den Bogaard MJ, Maas S, Hamel B, Salieb-Beugelaar G, Simon M, Letteboer T, van der Smagt J, Kroes H, Mannens M. Hypomethylation of the H19 gene causes not only Silver-Russell syndrome (SRS) but also isolated

asymmetry or an SRS-like phenotype. Am J Hum Genet 2006; 78(4):604–14.
[74] Douc-Rasy S, Barrois M, Fogel S, Ahomadegbe JC, Stehelin D, Coll J, Riou G. High incidence of loss of heterozygosity and abnormal imprinting of H19 and IGF2 genes in invasive cervical carcinomas. Uncoupling of H19 and IGF2 expression and biallelic hypomethylation of H19. Oncogene 1996;12(2):423–30.
[75] Steenman MJ, Rainier S, Dobry CJ, Grundy P, Horon IL, Feinberg AP. Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumour. Nat Genet 1994;7(3):433–9.
[76] Bliek J, Maas SM, Ruijter JM, Hennekam RC, Alders M, Westerveld A, Mannens MM. Increased tumour risk for BWS patients correlates with aberrant H19 and not KCNQ1OT1 methylation: occurrence of KCNQ1OT1 hypomethylation in familial cases of BWS. Hum Mol Genet 2001;10(5):467–76.
[77] Higashimoto K, Soejima H, Saito T, Okumura K, Mukai T. Imprinting disruption of the CDKN1C/KCNQ1OT1 domain: the molecular mechanisms causing Beckwith-Wiedemann syndrome and cancer. Cytogenet Genome Res 2006;113(1–4): 306–12.
[78] Robbins KM, Stabley DL, Holbrook J, Sahraoui R, Sadreameli A, Conard K, Baker L, Gripp KW, Sol-Church K. Paternal uniparental disomy with segmental loss of heterozygosity of chromosome 11 are hallmark characteristics of syndromic and sporadic embryonal rhabdomyosarcoma. Am J Med Genet A 2016;170(12):3197–206.
[79] St. Jude Children's Research Hospital-Washington University Pediatric Cancer Genome P Chen X, Stewart E, Shelat AA, Qu C, Bahrami A, Hatley M, Wu G, Bradley C, McEvoy J, Pappo A, Spunt S, Valentine MB, Valentine V, Krafcik F, Lang WH, Wierdl M, Tsurkan L, Tolleman V, Federico SM, Morton C, Lu C, Ding L, Easton J, Rusch M, Nagahawatte P, Wang J, Parker M, Wei L, Hedlund E, Finkelstein D, Edmonson M, Shurtleff S, Boggs K, Mulder H, Yergeau D, Skapek S, Hawkins DS, Ramirez N, Potter PM, Sandoval JA, Davidoff AM, Mardis ER, Wilson RK, Zhang J, Downing JR, Dyer MA. Targeting oxidative stress in embryonal rhabdomyosarcoma. Cancer Cell 2013;24(6):710–24.
[80] Seki M, Nishimura R, Yoshida K, Shimamura T, Shiraishi Y, Sato Y, Kato M, Chiba K, Tanaka H, Hoshino N, Nagae G, Shiozawa Y, Okuno Y, Hosoi H, Tanaka Y, Okita H, Miyachi M, Souzaki R, Taguchi T, Koh K, Hanada R, Kato K, Nomura Y, Akiyama M, Oka A, Igarashi T, Miyano S, Aburatani H, Hayashi Y, Ogawa S, Takita J. Integrated genetic and epigenetic analysis defines novel molecular subgroups in rhabdomyosarcoma. Nat Commun 2015;6:7557.
[81] Casey DL, Wexler LH, Pitter KL, Samstein RM, Slotkin EK, Wolden SL. Genomic determinants of clinical outcomes in rhabdomyosarcoma. Clin Cancer Res 2020;26(5):1135–40.
[82] George SL, Izquierdo E, Campbell J, Koutroumanidou E, Proszek P, Jamal S, Hughes D, Yuan L, Marshall LV, Carceller F, Chisholm JC, Vaidya S, Mandeville H, Angelini P, Wasti A, Bexelius T, Thway K, Gatz SA, Clarke M, Al-Lazikani B, Barone G, Anderson J, Tweddle DA, Gonzalez D, Walker BA, Barton J, Depani S, Eze J, Ahmed SW, Moreno L, Pearson A, Shipley J, Jones C, Hargrave D, Jacques TS, Hubank M, Chesler L. A tailored molecular profiling programme for children with cancer to identify clinically actionable genetic alterations. Eur J Cancer 2019;121:224–35.
[83] Izquierdo E, Yuan L, George S, Hubank M, Jones C, Proszek P, Shipley J, Gatz SA, Stinson C, Moore AS, Clifford SC, Hicks D, Lindsey JC, Hill RM, Jacques TS, Chalker J, Thway K, O'Connor S, Marshall L, Moreno L, Pearson A, Chesler L, Walker BA, De Castro DG. Development of a targeted sequencing approach to identify prognostic, predictive and diagnostic markers in paediatric solid tumours. Oncotarget 2017; 8(67):112036–50.
[84] Shern JF, Selfe J, Izquierdo E, Patidar R, Chou HC, Song YK, Yohe ME, Sindiri S, Wei J, Wen X, Rudzinski ER, Barkauskas DA, Lo T, Hall D, Linardic CM, Hughes D, Jamal S, Jenney M, Chisholm J, Brown R, Jones K, Hicks B, Angelini P, George S, Chesler L, Hubank M, Kelsey A, Gatz SA, Skapek SX, Hawkins DS, Shipley JM, Khan J. Genomic classification and clinical outcome in rhabdomyosarcoma: a report from an international Consortium. J Clin Oncol 2021: JCO2003060.
[85] Shukla N, Ameur N, Yilmaz I, Nafa K, Lau CY, Marchetti A, Borsu L, Barr FG, Ladanyi M. Oncogene mutation profiling of pediatric solid tumors reveals significant subsets of embryonal rhabdomyosarcoma and neuroblastoma with mutated genes in growth signaling pathways. Clin Cancer Res 2012;18(3):748–57.
[86] the German Cooperative Soft Tissue Sarcoma S. Leuschner I, Langhans I, Schmitz R, Harms D, Mattke A, Treuner J, Kiel Pediatric Tumor R. p53 and mdm-2 expression in rhabdomyosarcoma of childhood and adolescence: clinicopathologic study by the Kiel pediatric tumor registry and the German cooperative soft tissue sarcoma study Pediatr Dev Pathol 2003;6(2):128–36.
[87] Ignatius MS, Hayes MN, Moore FE, Tang Q, Garcia SP, Blackburn PR, Baxi K, Wang L, Jin A, Ramakrishnan A, Reeder S, Chen Y, Nielsen GP, Chen EY, Hasserjian RP, Tirode F, Ekker SC, Langenau DM. tp53 deficiency causes a wide tumor spectrum and increases embryonal rhabdomyosarcoma metastasis in zebrafish, vol. 7. Elife; 2018.
[88] Van Antwerp ME, Chen DG, Chang C, Prochownik EV. A point mutation in the MyoD basic domain imparts c-Myc-like properties. Proc Natl Acad Sci U S A 1992;89(19):9010–4.
[89] Kohsaka S, Shukla N, Ameur N, Ito T, Ng CK, Wang L, Lim D, Marchetti A, Viale A, Pirun M, Socci ND, Qin LX, Sciot R, Bridge J, Singer S, Meyers P, Wexler LH, Barr FG, Dogan S, Fletcher JA, Reis-Filho JS, Ladanyi M. A recurrent neomorphic mutation in MYOD1 defines a clinically aggressive subset of embryonal rhabdomyosarcoma associated with PI3K-AKT pathway mutations. Nat Genet 2014;46(6):595–600.
[90] Rekhi B, Upadhyay P, Ramteke MP, Dutt A. MYOD1 (L122R) mutations are associated with spindle cell and sclerosing rhabdomyosarcomas with aggressive clinical outcomes. Mod Pathol 2016;29(12):1532–40.
[91] Agaram NP, LaQuaglia MP, Alaggio R, Zhang L, Fujisawa Y, Ladanyi M, Wexler LH, Antonescu CR. MYOD1-mutant spindle cell and sclerosing rhabdomyosarcoma: an aggressive subtype irrespective of age. A reappraisal for molecular classification and risk stratification. Mod Pathol 2019;32(1):27–36.
[92] Ahmed AA, Habeebu S, Farooqi MS, Gamis AS, Gonzalez E, Flatt T, Sherman A, Surrey L, Arnold MA, Conces M, Koo S, Dioufa N, Barr FG, Tsokos MG. MYOD1 as a prognostic indicator in rhabdomyosarcoma. Pediatr Blood Cancer 2021;68(9): e29085.
[93] Barr FG, Duan F, Smith LM, Gustafson D, Pitts M, Hammond S, Gastier-Foster JM. Genomic and clinical analyses of 2p24 and 12q13-q14 amplification in alveolar rhabdomyosarcoma: a report from the Children's Oncology Group. Genes Chromosomes Cancer 2009;48(8):661–72.
[94] Hachitanda Y, Toyoshima S, Akazawa K, Tsuneyoshi M. N-myc gene amplification in rhabdomyosarcoma detected by fluorescence in situ hybridization: its correlation with histologic features. Mod Pathol 1998;11(12):1222–7.
[95] Williamson D, Lu YJ, Gordon T, Sciot R, Kelsey A, Fisher C, Poremba C, Anderson J, Pritchard-Jones K, Shipley J. Relationship between MYCN copy number and expression in rhabdomyosarcomas and correlation with adverse prognosis in the alveolar subtype. J Clin Oncol 2005; 23(4):880–8.
[96] Klega K, Imamovic-Tuco A, Ha G, Clapp AN, Meyer S, Ward A, Clinton C, Nag A, Van Allen E, Mullen E, DuBois SG, Janeway K, Meyerson M, Thorner AR, Crompton BD.

Detection of somatic structural variants enables quantification and characterization of circulating tumor DNA in children with solid tumors. JCO Precis Oncol 2018;2018.

[97] Lennon AM, Buchanan AH, Kinde I, Warren A, Honushefsky A, Cohain AT, Ledbetter DH, Sanfilippo F, Sheridan K, Rosica D, Adonizio CS, Hwang HJ, Lahouel K, Cohen JD, Douville C, Patel AA, Hagmann LN, Rolston DD, Malani N, Zhou S, Bettegowda C, Diehl DL, Urban B, Still CD, Kann L, Woods JI, Salvati ZM, Vadakara J, Leeming R, Bhattacharya P, Walter C, Parker A, Lengauer C, Klein A, Tomasetti C, Fishman EK, Hruban RH, Kinzler KW, Vogelstein B, Papadopoulos N. Feasibility of blood testing combined with PET-CT to screen for cancer and guide intervention. Science 2020;(6499):369.

[98] Weiser DA, West-Szymanski DC, Fraint E, Weiner S, Rivas MA, Zhao CWT, He C, Applebaum MA. Progress toward liquid biopsies in pediatric solid tumors. Cancer Metastasis Rev 2019;38(4):553–71.

[99] Eguchi-Ishimae M, Tezuka M, Kokeguchi T, Nagai K, Moritani K, Yonezawa S, Tauchi H, Tokuda K, Ishida Y, Ishii E, Eguchi M. Early detection of the PAX3-FOXO1 fusion gene in circulating tumor-derived DNA in a case of alveolar rhabdomyosarcoma. Genes Chromosomes Cancer 2019;58(8):521–9.

[100] Stegmaier S, Sparber-Sauer M, Aakcha-Rudel E, Munch P, Reeh T, Feuchtgruber S, Hallmen E, Blattmann C, Bielack S, Klingebiel T, Koscielniak E. Fusion transcripts as liquid biopsy markers in alveolar rhabdomyosarcoma and synovial sarcoma: a report of the Cooperative Weichteilsarkom Studiengruppe (CWS). Pediatr Blood Cancer 2022:e29652.

[101] Ghayad SE, Rammal G, Ghamloush F, Basma H, Nasr R, Diab-Assaf M, Chelala C, Saab R. Exosomes derived from embryonal and alveolar rhabdomyosarcoma carry differential miRNA cargo and promote invasion of recipient fibroblasts. Sci Rep 2016;6:37088.

[102] Lak NSM, Voormanns TL, Zappeij-Kannegieter L, van Zogchel LMJ, Fiocco M, van Noesel MM, Merks JHM, van der Schoot CE, Tytgat GAM, Stutterheim J. Improving risk stratification for pediatric patients with rhabdomyosarcoma by molecular detection of disseminated disease. Clin Cancer Res 2021;27(20):5576–85.

[103] Poli E, Zin A, Cattelan M, Tombolan L, Zanetti I, Scagnellato A, Bonvini P, Bisogno G. Prognostic value of circulating IGFBP2 and related autoantibodies in children with metastatic rhabdomyosarcomas. Diagnostics 2020;10(2).

[104] Tombolan L, Millino C, Pacchioni B, Cattelan M, Zin A, Bonvini P, Bisogno G. Circulating miR-26a as potential prognostic biomarkers in pediatric rhabdomyosarcoma. Front Genet 2020;11:606274.

[105] Tombolan L, Rossi E, Zin A, Santoro L, Bonvini P, Zamarchi R, Bisogno G. Pediatric sarcomas display a variable EpCAM expression in a histology-dependent manner. Transl Oncol 2020;13(11):100846.

[106] Greytak SR, Engel KB, Parpart-Li S, Murtaza M, Bronkhorst AJ, Pertile MD, Moore HM. Harmonizing cell-free DNA collection and processing practices through evidence-based guidance. Clin Cancer Res 2020;26(13):3104–9.

[107] Soliman H, Shah V, Srkalovic G, Mahtani R, Levine E, Mavromatis B, Srinivasiah J, Kassar M, Gabordi R, Qamar R, Untch S, Kling HM, Treece T, Audeh W. MammaPrint guides treatment decisions in breast Cancer: results of the IMPACt trial. BMC Cancer 2020;20(1):81.

[108] Davicioni E, Finckenstein FG, Shahbazian V, Buckley JD, Triche TJ, Anderson MJ. Identification of a PAX-FKHR gene expression signature that defines molecular classes and determines the prognosis of alveolar rhabdomyosarcomas. Cancer Res 2006;66(14):6936–46.

[109] Davicioni E, Anderson JR, Buckley JD, Meyer WH, Triche TJ. Gene expression profiling for survival prediction in pediatric rhabdomyosarcomas: a report from the children's oncology group. J Clin Oncol 2010;28(7):1240–6.

[110] Hingorani P, Missiaglia E, Shipley J, Anderson JR, Triche TJ, Delorenzi M, Gastier-Foster J, Wing M, Hawkins DS, Skapek SX. Clinical application of prognostic gene expression signature in fusion gene-negative rhabdomyosarcoma: a report from the children's oncology group. Clin Cancer Res 2015;21(20):4733–9.

[111] Ferrari A, Ianno MF, Carenzo A, Fortunato O, Casanova M, Chiaravalli S, Bergamaschi L, Bertulli R, Cattaneo F, Collini P, Trama A, Sozzi G, Massimino M, De Cecco L, Gasparini P. Complexity index in sarcoma and genomic grade index gene signatures in rhabdomyosarcoma of pediatric and adult ages. Pediatr Blood Cancer 2021;68(7):e28987.

[112] Chibon F, Lagarde P, Salas S, Perot G, Brouste V, Tirode F, Lucchesi C, de Reynies A, Kauffmann A, Bui B, Terrier P, Bonvalot S, Le Cesne A, Vince-Ranchere D, Blay JY, Collin F, Guillou L, Leroux A, Coindre JM, Aurias A. Validated prediction of clinical outcome in sarcomas and multiple types of cancer on the basis of a gene expression signature related to genome complexity. Nat Med 2010;16(7):781–7.

[113] Lesluyes T, Chibon F. A global and integrated analysis of CINSARC-associated genetic defects. Cancer Res 2020;80(23):5282–90.

[114] Olanich ME, Barr FG. A call to ARMS: targeting the PAX3-FOXO1 gene in alveolar rhabdomyosarcoma. Expert Opin Ther Targets 2013;17(5):607–23.

[115] Dietz KN, Miller PJ, Hollenbach AD. Phosphorylation of serine 205 by the protein kinase CK2 persists on Pax3-FOXO1, but not Pax3, throughout early myogenic differentiation. Biochemistry 2009;48(49):11786–95.

[116] Liu L, Wu J, Ong SS, Chen T. Cyclin-dependent kinase 4 phosphorylates and positively regulates PAX3-FOXO1 in human alveolar rhabdomyosarcoma cells. PLoS One 2013;8(2):e58193.

[117] Ommer J, Selfe JL, Wachtel M, O'Brien EM, Laubscher D, Roemmele M, Kasper S, Delattre O, Surdez D, Petts G, Kelsey A, Shipley J, Schafer BW. Aurora A kinase inhibition destabilizes PAX3-FOXO1 and MYCN and synergizes with navitoclax to induce rhabdomyosarcoma cell death. Cancer Res 2020;80(4):832–42.

[118] Thalhammer V, Lopez-Garcia LA, Herrero-Martin D, Hecker R, Laubscher D, Gierisch ME, Wachtel M, Bode P, Nanni P, Blank B, Koscielniak E, Schafer BW. PLK1 phosphorylates PAX3-FOXO1, the inhibition of which triggers regression of alveolar Rhabdomyosarcoma. Cancer Res 2015;75(1):98–110.

[119] Bharathy N, Berlow NE, Wang E, Abraham J, Settelmeyer TP, Hooper JE, Svalina MN, Bajwa Z, Goros MW, Hernandez BS, Wolff JE, Pal R, Davies AM, Ashok A, Bushby D, Mancini M, Noakes C, Goodwin NC, Ordentlich P, Keck J, Hawkins DS, Rudzinski ER, Mansoor A, Perkins TJ, Vakoc CR, Michalek JE, Keller C. Preclinical rationale for entinostat in embryonal rhabdomyosarcoma. Skelet Muscle 2019;9(1):12.

[120] Gryder BE, Pomella S, Sayers C, Wu XS, Song Y, Chiarella AM, Bagchi S, Chou HC, Sinniah RS, Walton A, Wen X, Rota R, Hathaway NA, Zhao K, Chen J, Vakoc CR, Shern JF, Stanton BZ, Khan J. Histone hyperacetylation disrupts core gene regulatory architecture in rhabdomyosarcoma. Nat Genet 2019;51(12):1714–22.

[121] Gryder BE, Yohe ME, Chou HC, Zhang X, Marques J, Wachtel M, Schaefer B, Sen N, Song Y, Gualtieri A, Pomella S, Rota R, Cleveland A, Wen X, Sindiri S, Wei JS, Barr FG, Das S, Andresson T, Guha R, Lal-Nag M, Ferrer M, Shern JF, Zhao K, Thomas CJ, Khan J. PAX3-FOXO1 establishes

myogenic super enhancers and confers BET bromodomain vulnerability. Cancer Discov 2017;7(8):884–99.
[122] Alijaj N, Moutel S, Gouveia ZL, Gray M, Roveri M, Dzhumashev D, Weber F, Meier G, Luciani P, Rossler JK, Schafer BW, Perez F, Bernasconi M. Novel FGFR4-targeting single-domain antibodies for multiple targeted therapies against rhabdomyosarcoma. Cancers 2020;12(11).
[123] Dyson KA, Stover BD, Grippin A, Mendez-Gomez HR, Lagmay J, Mitchell DA, Sayour EJ. Emerging trends in immunotherapy for pediatric sarcomas. J Hematol Oncol 2019;12(1):78.
[124] Xiao W, Wang J, Wen X, Xu B, Que Y, Yu K, Xu L, Zhao J, Pan Q, Zhou P, Zhang X. Chimeric antigen receptor-modified T-cell therapy for platelet-derived growth factor receptor alpha-positive rhabdomyosarcoma. Cancer 2020;126(9):2093–100.
[125] Long AH, Highfill SL, Cui Y, Smith JP, Walker AJ, Ramakrishna S, El-Etriby R, Galli S, Tsokos MG, Orentas RJ, Mackall CL. Reduction of MDSCs with all-trans retinoic acid improves CAR therapy efficacy for sarcomas. Cancer Immunol Res 2016;4(10):869–80.
[126] Chen L, Oke T, Siegel N, Cojocaru G, Tam AJ, Blosser RL, Swailes J, Ligon JA, Lebid A, Morris C, Levin A, Rhee DS, Johnston FM, Greer JB, Meyer CF, Ladle BH, Thompson ED, Montgomery EA, Choi W, McConkey DJ, Anders RA, Pardoll DM, Llosa NJ. The immunosuppressive niche of soft-tissue sarcomas is sustained by tumor-associated macrophages and characterized by intratumoral tertiary lymphoid structures. Clin Cancer Res 2020;26(15):4018–30.
[127] Pandey PR, Chatterjee B, Olanich ME, Khan J, Miettinen MM, Hewitt SM, Barr FG. PAX3-FOXO1 is essential for tumour initiation and maintenance but not recurrence in a human myoblast model of rhabdomyosarcoma. J Pathol 2017;241(5):626–37.
[128] Chen X, Makarewicz JM, Knauf JA, Johnson LK, Fagin JA. Transformation by Hras(G12V) is consistently associated with mutant allele copy gains and is reversed by farnesyl transferase inhibition. Oncogene 2014;33(47):5442–9.
[129] Canon J, Rex K, Saiki AY, Mohr C, Cooke K, Bagal D, Gaida K, Holt T, Knutson CG, Koppada N, Lanman BA, Werner J, Rapaport AS, San Miguel T, Ortiz R, Osgood T, Sun JR, Zhu X, McCarter JD, Volak LP, Houk BE, Fakih MG, O'Neil BH, Price TJ, Falchook GS, Desai J, Kuo J, Govindan R, Hong DS, Ouyang W, Henary H, Arvedson T, Cee VJ, Lipford JR. The clinical KRAS(G12C) inhibitor AMG 510 drives anti-tumour immunity. Nature 2019;575(7781):217–23.
[130] Fell JB, Fischer JP, Baer BR, Ballard J, Blake JF, Bouhana K, Brandhuber BJ, Briere DM, Burgess LE, Burkard MR, Chiang H, Chicarelli MJ, Davidson K, Gaudino JJ, Hallin J, Hanson L, Hee K, Hicken EJ, Hinklin RJ, Marx MA, Mejia MJ, Olson P, Savechenkov P, Sudhakar N, Tang TP, Vigers GP, Zecca H, Christensen JG. Discovery of tetrahydropyridopyrimidines as irreversible covalent inhibitors of KRAS-G12C with in vivo activity. ACS Med Chem Lett 2018;9(12):1230–4.
[131] Ryan MB, Fece de la Cruz F, Phat S, Myers DT, Wong E, Shahzade HA, Hong CB, Corcoran RB. Vertical pathway inhibition overcomes adaptive feedback resistance to KRAS(G12C) inhibition. Clin Cancer Res 2020;26(7):1633–43.
[132] Schoffski P, Wozniak A, Leahy MG, Aamdal S, Rutkowski P, Bauer S, Richter S, Grunwald V, Debiec-Rychter M, Sciot R, Geoerger B, Marreaud S, Collette S, Nzokirantevye A, Strauss SJ. The tyrosine kinase inhibitor crizotinib does not have clinically meaningful activity in heavily pre-treated patients with advanced alveolar rhabdomyosarcoma with FOXO rearrangement: European Organisation for Research and Treatment of Cancer phase 2 trial 90101 'CREATE'. Eur J Cancer 2018;94:156–67.
[133] Schuetze SM, Wathen JK, Lucas DR, Choy E, Samuels BL, Staddon AP, Ganjoo KN, von Mehren M, Chow WA, Loeb DM, Tawbi HA, Rushing DA, Patel SR, Thomas DG, Chugh R, Reinke DK, Baker LH. SARC009: phase 2 study of dasatinib in patients with previously treated, high-grade, advanced sarcoma. Cancer 2016;122(6):868–74.
[134] Wagner LM, Fouladi M, Ahmed A, Krailo MD, Weigel B, DuBois SG, Doyle LA, Chen H, Blaney SM. Phase II study of cixutumumab in combination with temsirolimus in pediatric patients and young adults with recurrent or refractory sarcoma: a report from the Children's Oncology Group. Pediatr Blood Cancer 2015;62(3):440–4.
[135] Renshaw J, Taylor KR, Bishop R, Valenti M, De Haven Brandon A, Gowan S, Eccles SA, Ruddle RR, Johnson LD, Raynaud FI, Selfe JL, Thway K, Pietsch T, Pearson AD, Shipley J. Dual blockade of the PI3K/AKT/mTOR (AZD8055) and RAS/MEK/ERK (AZD6244) pathways synergistically inhibits rhabdomyosarcoma cell growth in vitro and in vivo. Clin Cancer Res 2013;19(21):5940–51.
[136] Geyer N, Ridzewski R, Bauer J, Kuzyakova M, Dittmann K, Dullin C, Rosenberger A, Schildhaus HU, Uhmann A, Fulda S, Hahn H. Different response of ptch mutant and ptch wildtype rhabdomyosarcoma toward SMO and PI3K inhibitors. Front Oncol 2018;8:396.
[137] McKinnon T, Venier R, Yohe M, Sindiri S, Gryder BE, Shern JF, Kabaroff L, Dickson B, Schleicher K, Chouinard-Pelletier G, Menezes S, Gupta A, Zhang X, Guha R, Ferrer M, Thomas CJ, Wei Y, Davani D, Guidos CJ, Khan J, Gladdy RA. Functional screening of FGFR4-driven tumorigenesis identifies PI3K/mTOR inhibition as a therapeutic strategy in rhabdomyosarcoma. Oncogene 2018;37(20):2630–44.
[138] Blandino G, Di Agostino S. New therapeutic strategies to treat human cancers expressing mutant p53 proteins. J Exp Clin Cancer Res 2018;37(1):30.
[139] Stewart E, Federico SM, Chen X, Shelat AA, Bradley C, Gordon B, Karlstrom A, Twarog NR, Clay MR, Bahrami A, Freeman 3rd BB, Xu B, Zhou X, Wu J, Honnell V, Ocarz M, Blankenship K, Dapper J, Mardis ER, Wilson RK, Downing J, Zhang J, Easton J, Pappo A, Dyer MA. Orthotopic patient-derived xenografts of paediatric solid tumours. Nature 2017;549(7670):96–100.
[140] Olanich ME, Sun W, Hewitt SM, Abdullaev Z, Pack SD, Barr FG. CDK4 amplification reduces sensitivity to CDK4/6 inhibition in fusion-positive rhabdomyosarcoma. Clin Cancer Res 2015;21(21):4947–59.
[141] Hughes GR, Dudey AP, Hemmings AM, Chantry A. Frontiers in PROTACs. Drug Discov Today 2021;26(10):2377–83.