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Women in pediatric oncology vol II: 2022

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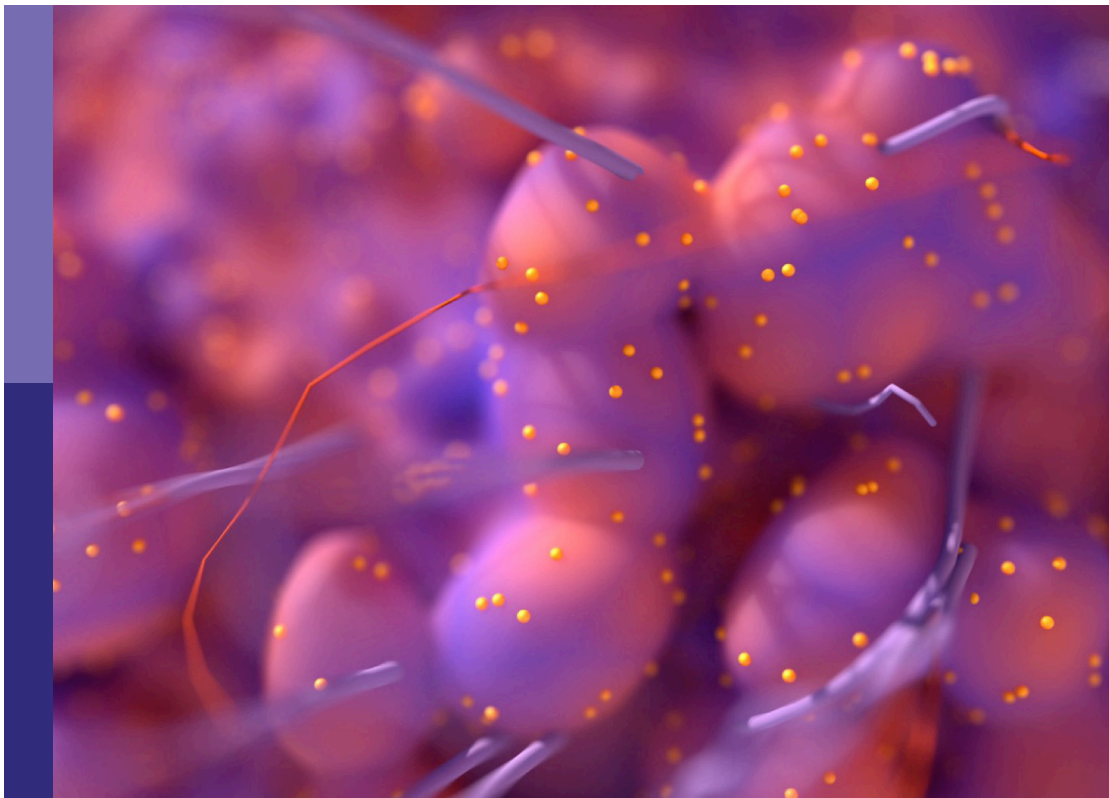
Published in
Frontiers in Oncology
Frontiers in Pediatrics



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ISSN 1664-8714
ISBN 978-2-8325-2968-3
DOI 10.3389/978-2-8325-2968-3

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Women in pediatric oncology vol II: 2022

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Citation

Kim, Y.-m., Kitlinska, J., eds. (2023). *Women in pediatric oncology vol II: 2022*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2968-3

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frontiers | Frontiers in Oncology

TYPE Editorial
PUBLISHED 26 June 2023
DOI 10.3389/fonc.2023.1223296

Check for updates

OPEN ACCESS

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RECEIVED 15 May 2023
ACCEPTED 06 June 2023
PUBLISHED 26 June 2023

CITATION
Kim Y-m and Kitlinska JB (2023) Editorial: Women in pediatric oncology Vol II: 2022. *Front. Oncol.* 13:1223296. doi: 10.3389/fonc.2023.1223296

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Editorial: Women in pediatric oncology Vol II: 2022

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KEYWORDS

pediatric oncology, neuroblastoma, survivorship, rhabdoid tumor, angiosarcoma, ovarian mass, pediatric-type diffuse gliomas, myeloid sarcoma

Editorial on the Research Topic
Women in pediatric oncology Vol II: 2022

120 years ago, in December 1903, Marie Curie became the first woman to win a Nobel Prize. Since then, women have made numerous contributions to all fields of science. Yet, despite this undisputable progress, female scientists still remain a minority. Hence, it is particularly important to highlight and promote their work. This edition of the Research Topic is devoted to women involved in childhood cancer research.

While pediatric cancer research has led to advances in our understanding of the biology of childhood cancers, and thereby contributed to advances in diagnosis, treatment and survivor psychosocial care, there remains an unmet need for improving patient outcomes and quality of life of the childhood cancer survivors. The second volume of "*Women in Pediatric Oncology*" contains 15 articles spanning a broad range of topics, starting from basic science through clinical research and survivorship care. Sorteberg et al. identify the activation of cyclin dependent kinase $p21^{Cip/Waf1}$ as a potential mechanism of chemoresistance in high-risk neuroblastoma and present preclinical data demonstrating efficacy of its inhibitor in combination with routine chemotherapeutics. Cervi et al. report a complete response to Trk inhibitor treatment in a patient with angiosarcoma carrying KHDRBS1-NTRK3 fusion gene, the first such case and a perfect example of precision-based medicine. A review paper by Cruz-Galvez et al. provides a comprehensive overview of retinoblastoma – the known facts and novel findings pertaining to this classic, genetically-driven pediatric malignancy.

Women are also pioneering novel technologies and treatment approaches. Two papers by Petrilli et al. and Miller et al. describe the use of state-of-the-art molecular profiling methods to characterize heterogeneity of rhabdoid tumors and pediatric-type diffuse high-grade gliomas, respectively. Other articles in this collection focus on clinical practice and outcomes research. Samborska et al. describe treatment outcomes in patients with myeloid sarcoma, while Puglisi et al. focus on the clinical characteristics of patients with combined neuroblastic tumors and neurofibromatosis type 1. Ariagno et al. present a timely study on the impact of prior COVID-19 infection on the risk of endothelial dysfunction in pediatric and adolescent patients undergoing hematopoietic cell transplants.

The clinical practice in pediatric oncology has to be tailored to children and often does not follow the same protocols as the care for adult patients. This problem is emphasized by Wang et al., who evaluate a diagnostic performance of imaging techniques in children with ovarian masses. On the other hand, Reschke et al. describe the development of clinical protocols aiming at improving multidisciplinary care of children with high-risk malignancies.

The last group of the manuscripts included in this Research Topic focuses on psychosocial issues associated with care for pediatric and adolescent cancer patients. These studies range from challenges in communication between health providers and patients and/or their families, as well as everyday difficulties facing the patients, their caregivers and educators (Burgers et al.; McLoone et al.; Otth et al.; Otth and Scheinemann; Rockwell et al.).

Altogether, this collection of outstanding 15 articles is a perfect example of the scope and variety of research performed by women scientists focusing on pediatric oncology and hematology. After reading the collection of these articles, the reader will appreciate the multi-faceted approach of current childhood cancer research, which remains a work in progress.

Author contributions

JK and Y-MK reviewed and summarized the manuscripts published in the Research Topic. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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ORIGINAL RESEARCH
published: 29 June 2022
doi: 10.3389/fonc.2022.894586

Silence in Conversations About Advancing Pediatric Cancer

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Background and Objectives: Skillful use of silence by clinicians can support patient-centered communication. However, what makes a period of silence feel meaningful is not well understood. This study aimed to characterize profound, skillful silences during difficult conversations between pediatric oncologists, children with advancing cancer, and their families.

Methods: We audio-recorded serial disease reevaluation discussions between pediatric oncologists, patients with high-risk cancer, and their families across 24 months or until death, whichever occurred first. Using an inductive process, we performed content analysis across all dialogue recorded at timepoints of disease progression to examine types of silence.

Results: 17 patient-parent dyads with disease progression yielded 141 recorded conversations. Inductive coding yielded a layered typology of silence, including "intentional silence" (≥5 seconds), "profound silence" (≥5 seconds following receipt of difficult information, juxtaposed with statements of shared understanding, emotion, or enlightenment), and "stacked silence" (series of silences juxtaposed within dialogue). Intentional silence lasting ≥5 seconds occurred 238 times in 35/49 "bad news" recordings; nearly half (103/238) of these silences were identified as profound silence, in which silences appeared to create space for processing, allowed for questions to emerge, and synergized with empathic and affirmational statements. In most cases, profound silences involved the juxtaposition, or stacking, of multiple silences close together.

Conclusions: Profound silences occur often during conversations about advancing pediatric cancer and share distinct characteristics. Opportunities exist to teach clinicians to use profound and stacked silences with intention during difficult conversations as a fundamental aspect of communication.

Keywords: silence, communication, pediatric, cancer, medical education

OPEN ACCESS

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Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

Received: *11 March 2022*
Accepted: *31 May 2022*
Published: *29 June 2022*

Citation:
Rockwell SL, Woods CL, Lemmon ME, Baker JN, Mack JW, Andes KL and Kaye EC (2022) Silence in Conversations About Advancing Pediatric Cancer. Front. Oncol. 12:894586. doi: 10.3389/fonc.2022.894586

Abbreviations: U-CHAT trial, Understanding Communication in Healthcare to Achieve Trust trial.

INTRODUCTION

Children with cancer and their families face physical, psychosocial, and spiritual distress across the illness course. Honest, direct communication between patients, families, and clinicians is essential for provision of holistic, person-centered care during this stressful time (1–5). Use of silence is recognized as an integral aspect of empathic communication; silence serves as a mechanism for conveying support and respect, facilitating reflection, and bearing witness (6). In the context of suffering and difficult conversations, certain types of silence can create pivotal moments of shared understanding, connection, and presence (7). The use of empathic and clear communication punctuated by meaningful silence can build therapeutic alliance, reduce stress, and improve stakeholders' perceptions of patient- and family-centered care (1).

Yet not all silence is equal. Silence defined as an absence of speech alone may entail awkward moments between clinicians and patients or be interpreted negatively by stakeholders (8). Within the field of communication science, researchers have examined differences between silences that engender connection, distance, or neutrality within patient-clinician encounters (7, 8). In general, connectional silences occur rarely, while silences that represent distance and neutrality are more common (7, 9). Silences that engender a sense of connection often feel profound and have been described within social sciences as "the stillness of listening to humanity". (10)

Within medical research, the qualities and impact of profound silence as a communication tool remain understudied. In the field of pediatric cancer specifically, few studies have examined the characteristics of meaningful periods of silence within clinical encounters (7–9). The U-CHAT (Understanding Communication in Healthcare to Achieve Trust) trial was designed to better understand patterns in prognostic communication across advancing illness. In this paper, data from the UCHAT trial were analyzed to characterize the frequency and nature of silence in conversations about disease progression in advancing pediatric cancer.

MATERIALS AND METHODS

This study was conceptualized and developed by an interdisciplinary team of pediatric oncology and palliative care clinicians and researchers in collaboration with an institutional Bereaved Parent Steering Council; it was approved by the Institutional Review Board at St. Jude Children's Research Hospital [U-CHAT (Pro00006473); approval date: 7/12/2016]. We present study methods and findings following the COREQ (COnsolidated Criteria for REporting Qualitative Research) checklist (**Supplemental Table 1**) (11).

Details about study recruitment, enrollment, and data collection processes were previously published (4, 12). Briefly, we enrolled a convenience sample of 33 children with high-risk cancer, their parents, and their primary pediatric oncologists at an academic pediatric cancer center.

Eligibility criteria and recruitment processes are summarized in **Table 1**. We followed patient-parent dyads prospectively and audio-recorded serial disease reevaluation discussions occurring in the clinic or hospital setting across each patient's illness course until death or 24 months from disease progression on study, whichever occurred first. Demographic and disease-related information were extracted from the electronic medical record.

To better understand the landscape of silence as a facet of communication during difficult conversations, we analyzed all conversations at timepoints of disease progression (i.e., "bad news" conversations). A research team representing medical and nursing disciplines across pediatric oncology and palliative medicine (**Supplemental Table 2**) first reviewed the literature on silence as a communication approach within cancer care. Finding little consensus for fundamentals of connectional silence in pediatric cancer care, we used an inductive approach

TABLE 1 | Eligibility criteria, recruitment, and informed consent processes.

Protocol domain	Study information
Eligibility Criteria	• Eligible oncologists: Primary oncologists providing medical care to solid tumor patients at the institution. • Eligible patients: Aged 0-30 years, solid tumor diagnosis with survival of ≤50% estimated by their primary oncologist, projected to have ≥ 2 future time points of disease reevaluations. • Eligible parents/guardians: Legal caregiver of eligible patient, aged ≥18 years, English language proficiency, planned to accompany patient to medical visits.
Recruitment & Informed Consent	• Eligible primary oncologists were introduced to the study by the Principal Investigator (PI). The PI individually sent emails to eligible oncologists to determine interest in participating; once interest was expressed, the PI met one-on-one with oncologists to describe the study and complete the informed consent process. No oncologists declined participation. • Eligible patient/parent dyads were identified by the research team through review of outpatient clinic schedules and institutional trial lists. The PI reviewed identified patients to determine those with overall survival estimated at 50% or less. A member of the research team then asked the patient's primary oncologist: "In your clinical judgement, would you estimate [patient name]'s overall survival at 50% or less?" Permission to approach eligible dyads was requested from the primary oncologist. Patient-parent dyads were approached by a member of the research team during a clinic visit to determine interest in participation. If interested, the study was described in detail. Dyadic enrollment required agreement from both patient and parent. Patients aged ≥12 years provided assent, and patients aged ≥18 years and parents provided consent. • Any other clinicians or individuals (e.g., family or friends of the patient) who planned to join a recorded conversation were approached by a member of the research team to learn about the study; following informed consent processes, verbal consent was obtained for the presence of study non-participants.

(13) to generate a typology for silences found within these conversations. Briefly, two researchers (S.R., E.K.) repetitively listened to audio-recordings, conducted memo-writing, and used raw data to inform development of codes, code definitions, and salient examples (14). Additional researchers (C.W., J.B.) reviewed recorded content and provided feedback in iterative cycles of codebook development. The codebook was pilot tested across several recordings representing various communication styles to identify areas of variance. We did not calculate interrater reliability, given theory suggesting that quantifying variances undervalues the interpretative mission of qualitative analysis (15). However, all analysts met regularly to review, discuss, and reconcile variances to achieve consensus, modifying the codebook when needed to improve dependability, confirmability, and credibility of independent codes (16).

The codebook was finalized following deep review of sufficient raw data to reach saturation, with no new concepts emerging from the recorded dialogue. Three levels of silence emerged from this process to comprise the codebook. First, intentional silence indicated an uninterrupted pause lasting at least 5 seconds; lapses in conversation in the context of transitions between topics or activities were excluded. This threshold was chosen following iterative memoing that revealed that pauses less than 5 seconds often were challenging to define conclusively as intentional. Second, profound silence emerged as a type of a silence in which the preceding or subsequent 60 seconds of dialogue included painful prognostic information, expressions of emotion, or shared sense of enlightenment. Third, stacked silence comprised a series of silence codes juxtaposed closely within dialogue, with fewer than 90 seconds between the end of one silence and the beginning of the following silence. Codes were not mutually exclusive, allowing for one or more codes to be applied to a given pause. The complete set of silence codes is presented in **Table 2**, alongside boundary examples of silences that met one criterion but not others to bolster understanding of the proposed taxonomy.

All processes were conducted within MAXQDA, a mixed methods data analysis software system (Verbi GMBH, Berlin, Germany) (17). Following codebook finalization, independent double coding of each recorded encounter was performed by two analysts (S.R., C.W.), with weekly meetings to review coding variances and third-party (E.K., J.B.) adjudication to reach consensus. Consistency in code segmentation was reviewed to ensure a standardized approach (S.R., C.W., E.K.). Content analysis of dialogue surrounding coded silence was conducted, identifying themes co-occurring with profound silence (S.R., E.K.) Quotations surrounding profound silence and stacked silence were examined to identify patterns in language, content, and timing of silence to generate themes (S.R., E.K.).

RESULTS

For the 17 patient-parent dyads who experienced advancing disease during the study period, 141 disease reevaluation conversations were audio-recorded, comprising approximately 2400 minutes of recorded dialogue. Of these, 49 recorded discussions occurred at a timepoint of disease progression and were subsequently analyzed. Participating patients were mostly female (64.7%) and white (88.2%); further participant demographic variables are presented in **Table 3**. A median of 7 medical discussions per patient were recorded (range 1-19). Most patients (14/17) died during the study period; 3 remained alive at 24 months. Data on patient-parent dyads who declined enrollment have been previously published; briefly 17% of

TABLE 2 | Inductive Silence Codebook.

Code	Definition
Intentional Silence	Code for any uninterrupted pause that is 5 seconds or longer; a pause with intention, not including transitions. *Boundary examples for silences that meet 5 second threshold criterion, but not intentional criterion:* -Non-verbal pauses with ancillary distraction sounds (e.g., shuffling papers, rustling sounds as someone moves from one location to another, etc.) -Non-verbal pauses that come after statements indicating the need for a momentary pause (e.g., "Give me a sec to look up these results," or "Let me just find the right spot on the image," etc.) -e.g., Oncologist: "I'm amazed that she's been doing so well and this stuff hasn't gotten worse. *[>5 seconds of* silence, punctuated by rustling food wrappers] Ok. Other questions?*" Patient: "No."*
Profound Silence	A coded silence in which, during the preceding or following 60 seconds, the following occurs: The provider shares "bad news" information (or references having just given this information) to patient/family related to scan results OR treatment options OR progression of disease OR goals of care OR prognosis **AND** at least one element below (before or after silence code): Statements about shared understanding/acknowledgement between provider and family **OR** Statements or expressions of enlightenment/catharsis. Can include provider responding to a question or making a statement that includes an element of truth-telling **OR** Expression of emotion by patient/family which preceded or followed statement by provider giving indication of shared emotions/recognition of expressed emotions. Can include provider invitation to continue expression of emotion. *Boundary example for silence that meets the intentional criterion, but not the criterion for profound silence:* *Oncologist: So my opinion is even if she hasn't shown marked improvement in the bony lesions, if her symptoms are actually really good … [>5 seconds of silence] … we may also be getting some control there as well.*
Stacked Silence	A series of **Silence** codes that are linked together as one segment of conversation, with no more than 90 seconds occurring between the end of one silence and the beginning of the following silence.

TABLE 3 | Participating patient, parent, and oncologist characteristics.

Variable	n (%)
Patient (n=17)	
Gender	
Female	11 (64.7)
Male	6 (35.3)
Race	
White	15 (88.2)
Black	1 (5.9)
Mixed	1 (5.9)
Ethnicity	
Hispanic	0 (0)
Non-Hispanic	17 (100)
Age at Diagnosis	
0-2 years	2 (11.8)
3-11 years	6 (35.3)
12-18 years	7 (41.2)
19+ years	2 (11.8)
Parent (n=17)	
Gender/Role	
Female/mother	14 (82.4)
Male/father	3 (17.6)
Pediatric Oncologist (n=6)	
Gender	
Female	3 (50)
Male	3 (50)
Race	
White	6 (100)
Black	0 (0)
Ethnicity	
Hispanic	0 (0)
Non-Hispanic	6 (100)
Years in Clinical Practice	
1-4 years	2 (33)
5-9 year	2 (33)
10-19 years	0
20+ years	2 (33)

approached dyads (n=7 dyads) did not enroll due to hesitation or refusal by either the patient (n=4) or parent (n=4). Although small numbers, refusal rates did not appear disproportionate by race or ethnicity (4, 12).

In 35 out of 49 conversations (71%), periods of intentional silence were identified 238 times. Nearly half of these silences (103/238) were dual coded as profound silence. Profound silence length ranged from 5-102 seconds. Below, we describe features of profound silence and themes identified within dialogue preceding (i.e., prompting) and following (i.e., emerging from) moments of profound silence.

Creating Space for Processing

Profound pauses seemed to create space for patients and families to absorb painful information. At times, after sharing difficult news (e.g., disease progression or relapse, poor prognosis, incurable illness), the oncologist would pause purposefully, as if to give the patient and family time to hear the message:

> "So right now, we are really just going to try to find a drug that could potentially work, the likelihood of that happening is relatively low, but we can try a variety of experimental agents to see if they can work against this tumor. [Patient: Could I die]? You could potentially die from this, yes. **Silence-21 seconds.**

Offering Opportunity for Questions

Silence also appeared to create opportunities for questions from patients or families, which may not have been voiced without a prolonged nonverbal space. For example, in the following conversation, the oncologist paused after affirming a family's goals of care. Out of the resulting silence, the parent was able to formulate a question. When the oncologist continued to remain silent, the parent was able to verbalize and complete the emotionally challenging question:

> "And you've been working on this so hard. And you deserve to do those things. You deserve to get to go to prom and go to Disney world and all of those kinds of things. And part of our goal should be to help you do that. Absolutely. [Parent: If we don't [do treatment] …] **Silence-6 seconds.** [Parent: Is she goin' to hurt]? **Silence-8 seconds**."

Welcoming Empathic Statements

Many profound silences included an empathic statement by the oncologist, often emerging from the silence. Just as silence invited emotional expression to emerge or persist, short empathic utterances also created space for audible emotion to be held. In one conversation, the oncologist sat in silence while a parent cried, then offered brief condolences before reentering silence:

> "But I wouldn't probably do that [go on a trip] until the radiation is done. [Parent: Yeah. After the radiation is finished]? Assuming she feels good enough, that would be the time to try that. **Silence-8 seconds**. [parent audibly crying] I'm sorry. **Silence-37 seconds**. [parent audibly crying]."

At other times, expressions of empathy preceded silence, first acknowledging the grief and then bearing witness: "*I'm sorry sweetheart, I wish I had better news for you today.* **Silence-10 seconds** *[Patient Audibly Crying].*" In a different conversation, another oncologist expressed empathy before entering silence, creating space for shared grief:

> "I know that she is very resilient and that she is very positive, and that she is probably in denial. Which is perfectly understandable. But I know she doesn't feel good. [Parent: I know she doesn't feel good. [audibly crying.]] She just doesn't look the way she normally does. [Grandparent: I think she has lost a lot of her fighting spirit too.] I'm sorry. **Silence-10 seconds**. [parent audibly crying]."

Emphasizing Affirmation

Within spaces of profound silence, oncologists offered statements to affirm or validate different choices about treatment and goals of care made by the patient or family. In many cases, affirming statements occurred at the onset of a profound silence, where the silence that followed the statement further reinforced the authenticity of the affirmation. For example, an oncologist validated a family's prior interest in shifting focus to quality of life:

> "There is even the choice to focus less on the [cancer] itself and focus more on you and how you're feeling every day when you get up. So that you know you're maximizing, you know you're maximizing the way that you feel and that you can get the most out of every day … And you know none of those are wrong choices, it's just a matter of what, at this point, what you feel is the most important goals for you … **Silence-26.7 seconds.** [patient audibly crying]."

Oncologists also used silence to affirm their role as a partner and supporter across the illness course:

> "We really want you to come up with that, come up with lists so we can together all make the best decisions for you guys. With you guys, not for you, it's with you. Okay? **Silence-7 seconds**. [parent audibly sniffling]. I'm sorry. **Silence-5 seconds**."

Stacking Silence

Multiple silences often occurred in close proximity with one another, and approximately three-quarters of profound silences within "bad news" conversations involved stacked silences. We found a wide range of stacking, from two distinct silences occurring within 90 seconds of dialogue to up to 12 distinct silences occurring within 8 minutes of dialogue. In most stacked silence moments, approximately one silence occurred per minute of conversation to generate a series, evoking a rhythmic pattern to the conversation. In many cases, each subsequent silence helped to advance the conversation into further exploration of difficult topics:

> "Would you like for us to talk to [patient's name]? Do you want to talk to her? Do you want her to talk? What would you like us to do? [Parent: Well, you decide amongst yourselves]. **Silence-5 seconds**. Can you tell me what you think would be the most appropriate thing to do?…If we say we are going to try something else then we are already committing to something we don't know if we are going to do or not. [Parent: Okay]. Would you like us to tell her that? Would you like to tell her that? I'll do whatever you want us to do. [Parent: Y'all can tell her]. **Silence-7 seconds**. I'm very sorry. **Silence-22 seconds**."

In some cases, multiple silences were stacked within a few seconds of each other, with minimal conversation between silences. This phenomenon most commonly occurred when the oncologist spoke in brief phrases and allowed silence to dominate. In these scenarios, empathic statements typically punctuated a longer segment of silence, as previously described. These empathic interjections from the oncologist between silences often involved reassurance or bearing witness, with subsequent silence encouraging emotional expressions from the patient or family.

> "What can I do for you? [Parent: You've done everything. I mean … [parent audibly crying]]. **Silence-25 seconds**. [parent audibly crying] You've done everything too. **Silence-54 seconds**. [parent audibly crying]. I'm so sorry. **Silence-9 seconds**. [parent audibly crying]."

We also identified a pattern where oncologists juxtaposed open-ended questions with stacked silences, offering an invitation or opportunity for the patient or family to emerge from silence and continue a conversation at their own pace:

> "I don't think she can be cured. There may be a very, very slim possibility of controlling it with something. But I do not believe there is a cure for her disease unfortunately. **Silence-60 seconds**. [parent audibly sighing] What is going through your mind now? What questions do you have for us? **Silence-20 seconds**. [parent audibly sniffling] [Parent: I guess I just have to talk to her dad …]. **Silence-8 seconds**. [parent audibly crying] [Parent: Every bit of the mom in me would lean toward the experimental … you know]. Of course, that's understandable."

DISCUSSION

High-quality communication between clinicians and patients and their families is essential to person-centered care, improving psychosocial outcomes while facilitating therapeutic alliance in pediatric oncology (5, 18–20). Although silence is considered a strategic aspect of communication, the landscape of silence during difficult pediatric cancer conversations remains understudied. In this paper, we identified periods of intentional silence occurring in more than two-thirds of difficult conversations about disease progression. Nearly half of intentional silences were recognized as meaningful, or profound, in their ability to create space for processing, allow questions to emerge, and acknowledge and affirm emotional expression. Interestingly, most profound silences also involved the stacking of multiple silences close together.

Patients and families place high value on their clinician's recognition and affirmation of their emotions during difficult conversations (21–24). Prior data demonstrate that clinicians

inconsistently respond to emotional cues and concerns expressed by patients and families (22, 25) and frequently verbally dominate conversations, affording less time for patients or families to speak or ask questions (26, 27). When clinicians speak less, however, more opportunities for emotional disclosure by patients and families manifest (22). Building upon these findings, this study suggests that skillful use of silence, particularly in synergy with empathic statements and stacking of silence, has the potential to foster meaning-making and connection between clinician, patient, and family.

Being purposeful about creating space for silence, however, does not necessarily encompass skillful use of silence. Intuitively, unintentional silences (e.g., shuffling papers, transitioning locations, etc.) do not offer opportunities for connection or meaning-making; one might think that intentional silences intrinsically create these opportunities. Yet inductive coding revealed distinct concepts showing intentional silence as a pattern distinct from other types of silence, suggesting that being purposeful (intentional) about silence does not necessarily make the silence meaningful.

In this study, profound and stacked silences appeared to facilitate a psychological space in which everyone in the room had an opportunity to sit together and process information. These data emphasize the importance of silence as a tool for encouraging processing of emotions as well as processing of cognitive information, both of which influence decision making processes (28). Intense emotional reactions, such as those elicited when hearing information about disease progression, can derail an individual's capacity for processing and rational decision making (29). People experiencing intense emotions (sometimes described as "hot" states) make different choices compared to people in "cold" states (30), and the difference between a hot vs. cold state can impact person-centered decision making around treatment options and end of life care choices (28). Skillful use of silence by clinicians may give patients and families space for processing, allowing for further conversation and decision making to occur in a less "hot" state.

We advocate for silence to be taught as an integral aspect of communication training for clinicians. When faced with emotional expression by patients or families, clinicians most often respond with provision of information, which in turn decreases space for further emotional disclosure (22). Existing communication training programs emphasize provision of empathic statements and emotional support, but the specific importance of silence as a communication strategy is less often described in the literature (31, 32). When mentioned, silence generally is presented as a tool for creating space immediately after the provision of bad news (33), and additional opportunities exist to develop and explore experiential learning techniques to teach clinicians to integrate and stack silence with purpose across medical dialogue as a fundamental communication strategy.

Importantly, not all profound silences may be interpreted positively by patients or families; at times, silence during profound moments may be a default reaction when clinicians do not know what to say. Further research is needed to examine the impact of profound silence and stacked silence on therapeutic alliance and shared decision-making between clinicians, patients with serious illness, and their families.

This study has several limitations, including single site design, convenience sampling of participants, and limited racial and ethnic representation. All eligible oncologists participated, comprising a range of styles and years of clinical practice, however they are not necessarily representative of all oncologists' practice styles and strategies. A few discussions were not recorded due to logistical issues or at the request of the participating patient or parent. Although missing data could influence synthesis and interpretation of silences, given saturation of themes across thousands of recorded minutes, several missing timepoints are less likely to impact synthesis of findings. Codes were inductively derived based on clinical experience and lacked input from patient and family perspectives, which is needed for further validation in future studies.

In summary, intentional integration of silence in conjunction with empathic statements may enhance processing of information and emotional expression, fostering a sense of connection and meaning-making during difficult conversations between oncologists and their patients/family. Stacking silence also may afford opportunities for engendering profound, connectional moments during challenging clinical encounters.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by St. Jude IRB. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SR conceptualized this study, carried out the analyses, drafted the initial manuscript, and revised the manuscript. CW collected data, carried out the analyses, and critically reviewed the manuscript for important intellectual content. ML assisted with analysis and reviewed and revised the manuscript. JB conceptualized and designed the study, supervised data collection, and critically reviewed the manuscript for important intellectual content. JM conceptualized and designed the study, assisted with analysis, and reviewed and revised the manuscript. KA conceptualized and designed the study, supervised analysis, and critically reviewed the manuscript for important intellectual content. EK conceptualized and designed the study, coordinated and supervised data collection, carried out the analysis, drafted

the initial manuscript, and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

FUNDING

This work is supported by EK's Career Development Award from the National Palliative Care Research Center and by ALSAC. Additionally, ML receives salary support from the National Institute of Neurological Disorders and Stroke (K23NS116453).

ACKNOWLEDGMENTS

The authors acknowledge Melanie Gattas, CRA-RN for her assistance with data organization, as well as the Quality of Life Research Division for its support of this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.894586/full#supplementary-material

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ORIGINAL RESEARCH
published: 07 July 2022
doi: 10.3389/fonc.2022.935373

Clinical Characteristics and Treatment Outcomes of Myeloid Sarcoma in Children: The Experience of the Polish Pediatric Leukemia and Lymphoma Study Group

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Introduction: Myeloid sarcoma (MS) is an extramedullary malignant tumor composed of immature myeloid cells. It occurs in patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), or chronic myeloid leukemia (CML). MS may coincide with disease diagnosis or precede bone marrow involvement by months or even years; it can also represent the extramedullary manifestation of a relapse (1, 2).

Aim: The aim of this study is to describe clinical characteristics of children diagnosed with MS in Poland as well as to analyze diagnostic methods, treatment, and outcomes including overall survival (OS), relapse-free survival (RFS), and event-free survival (EFS). The study also attempted to identify factors determining treatment outcomes.

Patients: The study group comprised 43 patients (F=18, M=25) aged 0-18 years (median age, 10.0 years; mean age, 8.8 years) diagnosed with MS based on tumor biopsy and immunohistochemistry or identification of underlying bone marrow disease and extramedullary tumor according to imaging findings.

OPEN ACCESS

Edited by:
Simone Cesaro, Integrated University Hospital Verona, Italy

Reviewed by:
Daisuke Tomizawa, National Center for Child Health and Development (NCCHD), Japan
Jose Luis Lopez Lorenzo, University Hospital Fundación Jiménez Díaz, Spain

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Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

Received: *03 May 2022*
Accepted: *07 June 2022*
Published: *07 July 2022*

Citation:
Samborska M, Barańska M, Wachowiak J, Skalska-Sadowska J, Thambyrajah S, Czogała M, Balwierz W, Kołtan S, Peszyńska-Żelazny, Wysocki M, Ociepa T, Urasiński T, Wróbel G, Węcławek-Tompol J, Ukielska B, Chybicka A, Kitszel A, Krawczuk-Rybak M, Szmydki-Baran A, Malinowska I, Matysiak M, Mizia-Malarz A, Tomaszewska R, Szczepański T, Chodała-Grzywacz A, Karolczyk G, Maciejka-Kembłowska L, Irga-Jaworska N, Badowska W, Dopierała M, Kurzawa P and Derwich K (2022) Clinical Characteristics and Treatment Outcomes of Myeloid Sarcoma in Children: The Experience of the Polish Pediatric Leukemia and Lymphoma Study Group. Front. Oncol. 12:935373. doi: 10.3389/fonc.2022.935373

Methods: The clinical data and diagnostic and therapeutic methods used in the study group were analyzed. A statistical analysis of the treatment outcomes was conducted with STATISTICA v. 13 (StatSoft, Inc., Tulsa, OK, USA) and analysis of survival curves was conducted with MedCalc 11.5.1 (MedCalc Software, Ostend, Belgium). Statistical significance was considered at $p<0.05$.

Results: In the study group, MS was most frequently accompanied by AML. The most common site of involvement was skin, followed by orbital region. Skin manifestation of MS was more common in the age group <10 years. The most frequent genetic abnormality was the t(8;21)(q22;q22) translocation. The 5-year OS probability (pOS), 5-year RFS probability (pRFS), and 5-year EFS probability (pEFS) were 0.67 ± 0.08, 0.79 ± 0.07, and 0.65 ± 0.08, respectively. In patients with isolated MS and those with concurrent bone marrow involvement by AML/MDS, pOS values were 0.56 ± 0.12 and 0.84 ± 0.09 (p=0.0251), respectively, and pEFS values were 0.56 ± 0.12 and 0.82 ± 0.08 (p=0.0247), respectively. In patients with and without the t(8;21)(q22;q22) translocation, pEFS values were 0.90 ± 0.09 and 0.51 ± 0.14 (p=0.0490), respectively.

Conclusions: MS is a disease with a highly variable clinical course. Worse treatment outcomes were observed in patients with isolated MS compared to those with concurrent bone marrow involvement by AML/MDS. Patients with the t(8;21)(q22;q22) translocation were found to have significantly higher pEFS. MS location, age group, chemotherapy regimen, surgery, and/or radiotherapy did not have a significant influence on treatment outcomes. Further exploration of prognostic factors in children with MS is indicated.

Keywords: children, myeloid sarcoma, acute myeloid leukemia, prognosis, treatment

INTRODUCTION

According to the World Health Organization (WHO) 2008 classification, myeloid sarcoma (MS) is categorized as an acute myeloid leukemia (AML)–related neoplasm. It results from proliferation of cells of one or more myeloid lineages that subsequently form an extramedullary tumor mass disrupting the surrounding tissue architecture. MS was firstly described by Burns in 1811, followed by King in 1853, who used the term chloroma due e to the green appearance of cells under microscopic imaging as a result of the presence of myeloperoxidase (1, 2). Other names used in past literature are granulocytic sarcoma, extramedullary myeloid cell tumor, and myeloblastoma (3, 4).

The etiology of MS remains unclear. One of the factors promoting blast survival in extramedullary tissues is the presence of natural barriers (blood-brain barrier and blood-testis barrier) that impede the penetration of chemotherapeutic agents to the central nervous system (CNS) and gonads (5). A variety of mechanisms are suspected to underlie enhanced blast migration to extramedullary tissues, including the formation of complexes of metalloproteinases and leukocyte integrins (6, 7) as well as interactions between specific chemokines and their receptors (8).

MS most frequently coincides with a diagnosis of AML; it presents concurrently with AML in 2-8% of adult patients and up to 40% of pediatric patients (3, 5). However, statistical data of pediatric patients are limited. An analysis by Kobayashi et al. reported that among 240 patients with AML, 23.3% had extramedullary disease at baseline. The study also included patients with MS with CNS involvement (9). An analysis by Dusenbery et al. showed that extramedullary manifestation at diagnosis was found in 10.9% of 1,832 patients (10). In a study by Johnston et al., MS was diagnosed in 99 of 1,459 patients (6.7%) (11).

Isolated MS without concurrent bone marrow involvement (*de novo* MS) poses certain diagnostic problems. In children, only ambiguous cases of isolated MS have been reported. According to the literature, the incidence of isolated MS is 2/1,000,000 in adults and 0.7/1,000,000 in children. Untreated isolated MS progresses to AML in 90% of cases and mean time from diagnosis to bone marrow involvement is approximately 10.5-11 months (4).

A major issue is an extramedullary relapse of MS experienced by 5-12% of patients after allogeneic hematopoietic stem cell transplantation (alloHSCT) (12). The factors that are likely to increase the risk of such a relapse include the presence of extramedullary locations in the disease course, AML M4 or M5 according to the French–American–British (FAB) classification, advanced disease at transplantation, and high-risk genetic factors such as t(8;21), inv(16) and KMT2A rearrangement (12–15).

Aim

A summary of the experience of Polish pediatric oncology centers regarding pediatric patients with MS is an important

step towards a deeper understanding of this rare disease. The aim of this study is to describe clinical characteristics of children diagnosed with MS in Poland as well as to analyze diagnostic methods, treatment, and outcomes, including overall survival (OS), relapse-free survival (RFS), and event-free survival (EFS). The study also seeks to identify factors influencing treatment outcomes.

METHODS

An Excel database was created to collect data on clinical features and treatment methods and outcomes. An event was defined as a relapse, progression, death, or second cancer. Complete remission (CR), partial remission (PR), and late remission (LR) were assessed according to the patient's AML therapeutic protocol. Moreover, PR was reported in the case of partial tumor regression, even if CR in the bone marrow was achieved.

Qualitative variables were presented as numbers (N) and percentages (%). For bivariate comparisons, χ^2 or the Fisher's exact test was applied. χ^2 test was also used for multivariate comparisons.

Quantitative variables were expressed as mean ± standard deviation (SD), minimum and maximum values, and median. Normal distribution of the variables was assessed by performing the Shapiro-Wilk test. Since most of the variables did not follow Gaussian distribution, all the analyses were conducted using non-parametric tests. Comparisons between the two groups were performed using the non-parametric Mann–Whitney U test for independent variables.

The Kaplan–Meier method was used to determine pOS, pRFS, and pEFS. The influence of various categories (age group, MS presentation, tumor location, t(8;21)(q22;q22) translocation, treatment regimen, alloHSCT, surgical treatment, and radiotherapy) on survival times (OS, EFS, RFS) was assessed using the logrank test.

A statistical analysis was conducted with STATISTICA v. 13 (StatSoft, Inc, Tulsa, OK, USA) and analysis of survival curves was conducted with MedCalc 11.5.1 (MedCalc Software, Ostend, Belgium). Statistical significance was considered at $p<0.05$.

Patients

The study group comprised 43 patients with MS (F=18, M=25) aged 0-18 years (median, 10.0 years; mean, 8.8 years) who were hospitalized in 12 pediatric oncology and hematology centers in Poland between 1998 and 2019. Inclusion criteria were as follows: age from 0 to 19 years and diagnosis of MS based on histopathology and immunohistochemistry or clinical presentation in those patients with an extramedullary tumor and concurrent proliferative disease in the bone marrow such as AML, myelodysplastic syndrome (MDS), or chronic myeloid leukemia (CML).

For research purposes, patients were divided into two age groups: <10 years (n=21) and ≥10 years (n=22).

RESULTS

1. Study Group Characteristics

Diagnosis of Underlying Disease and Clinical Presentation of MS

In the study group, patients with AML predominated (29), twelve patients had isolated MS without bone marrow involvement, one patient was diagnosed with CML, and one patient was diagnosed with MDS.

The following disease presentations were reported in the study group (**Figure 1**):

- Presentation with bone marrow involvement by AML/MDS/CML – 24 patients (55.80%)
- *De novo* presentation in patients without bone marrow involvement **at diagnosis** – 16 patients (37.20%)
- Isolated MS relapse – 1 patient (2.32%)
- Mixed (medullary and extramedullary) relapse of MS – 2 patients (4.65%)

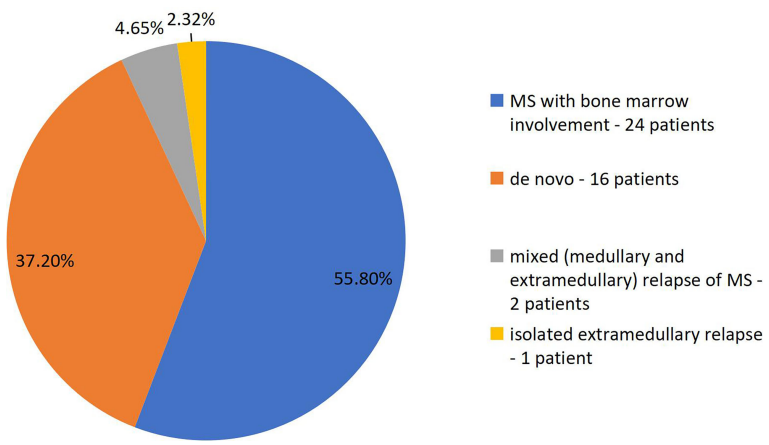

FIGURE 1 | Clinical presentations of myeloid sarcoma depending on the time relation to bone marrow involvement.

Among patients with CNS involvement, there was one patient (2%) with CNS-2 status and twelve patients (28%) with CNS-3 status. The absence of CNS involvement (CNS-1) was found in twenty-six patients (60%), while in four patients, CNS status was not assessed (no data).

Location and Symptoms

Sites of MS in the study group and symptom characteristics are presented in **Table 1**. The most common site was the skin (15 patients, 34.88%), followed by the orbital region (11 patients; 25.58%). Twenty-eight patients (65.11%) had other anatomical areas involved. In ten patients, MS occurred in two or more sites.

A significant relationship was found between the age groups and skin manifestation of MS (skin manifestation was significantly more frequent in patients <10 years of age; **p=0.0268**). In the group of childern with skin manifestation, 7 of 15 (46%) patients were at the age below 12 months.

Symptoms of MS depended on lesion location and the extent of the neoplasm.

Genetic Test Results in the Study Group

The most frequent mutation, detected in ten patients (23.25%), was the t(8;21)(q22;q22) translocation. Four patients (9.30%) had KMT2A gene rearrangement. Other aberrations were found in six patients. All the genetic test results of the patients are presented in **Table 2**.

Treatment

All the patients (43, 100%) received systemic chemotherapy. In 24 patients (56%), radiotherapy was administered as a part of the treatment; 15 patients (35%) received CNS radiotherapy according to the AML therapeutic regimen, 7 patients (16%) received radiotherapy to the tumor area (with doses of 18-30 Gy), and 2 patients (5%) were irradiated to CNS as well as MS area (with dose of 18 Gy). Six patients (14%) underwent surgical treatment. Nine patients (21%) were treated with alloHSCT. Treatment methods are shown in **Table 3**.

Chemotherapy Regimens

First-line chemotherapy regimen was usually based on current treatment guidelines for AML in children; patients were treated according to the AML-BFM 2004 Interim protocol (21 patients, 49%), AML-BFM 2012 protocol (10 patients, 23%), and AML-PPLLSG 98 protocol (10 patients, 23%) (17, 18). Two patients received treatment regimens other than those listed above. Of them, one patient (diagnosed with CML, isolated extramedullary relapse) received imatinib followed by idarubicin in combination with fludarabin (Ida-Fla) and, after complete tumor resection, low-intensity consolidation according to the AML-BFM 2001 protocol

TABLE 1 | Location of all MS sites in the study group and symptom characteristics.

Location	Symptom	n	n [%]
Skin (15/24.59%)	Nodules	**7**	**16.27%**
	Bluish lesions	**1**	**2.32%**
Orbital region (11/18.03%)	Exophthalmos	**5**	**11.62%**
	Orbital soft tissue swelling	**2**	**4.65%**
	Ptosis	**1**	**2.32%**
	Facial nerve paralysis	**2**	**4.65%**
	No symptoms (orbital involvement found with imaging tests)	**3**	**6.97%**
Other (35/57.38%)	Visible swelling of the affected area/tumor	**8**	**18.6%**
Paravertebral area (5/8.19%)	Fatigue	**8**	**18.6%**
Pelvis (3/4.91%)	Pain	**7**	**16.27%**
Skull (3/4.91%)	Fever	**5**	**11.62%**
CNS (3/4.91%)	Weight loss	**4**	**9.3%**
Lungs (2/3.27%)	Swollen lymph nodes	**4**	**9.3%**
Pleura (2/3.27%)	7th nerve paralysis	**4**	**9.3%**
Abdominal cavity (2/3.27%)	Hearing loss	**3**	**6.97%**
Liver (2/3.27%)	Symptoms of respiratory tract infection	**3**	**6.97%**
Middle ear (2/3.27%)	Paresis	**3**	**6.97%**
Femur (2/3.27%)	Paleness	**2**	**4.65%**
Cheek (1/1.63%)	Dyspnea	**2**	**4.65%**
Elbow joint (1/1.63%)	Joint pain	**1**	**2.32%**
Mammary glands (1/1.63%)	Apathy	**1**	**2.32%**
Gums (1/1.63%)	Horner's syndrome	**1**	**2.32%**
Nasopharynx (1/1.63%)	Petechiae	**1**	**2.32%**
Intracanal (1/1.63%)	Sweating	**1**	**2.32%**
Maxillary sinus (1/1.63%)	Hepatosplenomegaly	**1**	**2.32%**
Mediastinum (1/1.63%)	Abdominal distension	**1**	**2.32%**
Lymph nodes (1/1.63%)	Constipation	**1**	**2.32%**
	Anuria	**1**	**2.32%**
	Excessive growth of the head circumference	**1**	**2.32%**
	Otitis	**1**	**2.32%**

Number of locations.

TABLE 2 | The results of all genetic tests of bone marrow* in patients from the study group (n=43).

No.	Genetic test result
1	The t(8;21) translocation with AML1/ETO fusion gene on chromosome 8 and loss of the sex chromosome Y, characteristic of AML M2, were found in all metaphases. In addition, complex chromosome aberrations involving chromosomes 11 and 12 were found. BCR/ABL1 - negative. ITD-FLT3 - negative, NPM1 - negative, PML-RARA negative, inv 16 negative.
2	No PML/RARA fusion gene was found, indicating t(15;17) translocation. BCR/ABL fusion gene indicating t(9;22) translocation was found in 13/200 interphase nuclei. No tandem duplication and D85 mutation of the *FLT3* gene were found.
3	In 200 interphase nuclei, no BCR/ABL1 fusion gene was found, indicating t(9;22) translocation. Amplification of the MYCN oncogene over 10 copies was present in 26% of nuclei. 2010-03-02 Karyotype: 46,XY (16) In 200 interphase nuclei, no AML1/ETO fusion gene was found, indicating t(8;21) translocation. In 200 interphase nuclei, no inversion of chromosome 16 was found. No tandem duplication and D85 mutation of the *FLT3* gene were found. In 200 interphase nuclei, no PML/RARA fusion gene was found, indicating t(15;17) translocation. NCN=6400 copies WT1/10 up to 4 copies of ABL.
4	In 90% (180/200) of interphase nuclei, a double AML1/ETO fusion gene was found, indicating t(8;21) translocation. No PML/RARA fusion gene was found, indicating t(15;17) translocation, TEL/AML1, and thus t(12;21), deletion of 11q23, and other rearrangements involving KMT2A, tandem duplication and D85 mutation of the *FLT3* gene, chromosome 5q deletion, BCR/ABL1 fusion gene, and thus the t(9;22) translocation, DEK/NUP214 fusion gene, and thus the t(6;9) translocation. Karyotype: 45 X,Y, t(8;21)(q22;q22).
5*	Normal bone marrow. Tumor tissue tests: *NPM1* positive, 19% of the tumor cells presented 3 signals of the ABL1 I BCR gene.
6	In 10% of interphase nuclei, trisomy of chromosome 8 was found. Cytogenetic analysis (GTG) – normal karyotype in all metaphases. In 40% of interphase nuclei, trisomy of chromosome 8 was found. NMYC amplification – not found.
7	Karyotype: 46 XX. BCR/aBL1 negative, TELAML t(12;21) negative, KMT2A (11q23) negative, *FLT3* negative, inversion 16 - negative, *WT1* – positive.
8	The result of DNA analysis for the most common mutations in the *WT1* and *NPM1* genes – not found. The result of the analysis of the 28 most common chromosomal rearrangements of prognostic significance in leukemia – detection of fusion gene KMT2A-AFF1 t(4;11)(q21;q23).
9	Trisomy 21, the most common chromosome rearrangements in AML – negative.
10	No data
11	Karyotype: 90-92,XXYY,der(4) (15)M-BCR/ABL - negative; KMT2A/AF4 - negative; TEL/AML1 - negative; AML/ETO - negative; m-BCR/ABL - negative; E2A/PBX1 - negative; CBFb-MYH11 - negative; FLT3-ITD. – negative.
12	Karyotype: 45,X,-Y, t (8;21) (14)/46,XY (3); RT-PCR assay: M bcr/abl (-), mbcr/abl (-)
13	t(8;21)(q22;q22)
14	No data
15	t8;21 (q22;q22)
16	No data
17	t(8;21) (FISH + PCR) in bone marrow. *NPM1* and *FLT3* mutations were not found.
18	The bone marrow cytogenetic mutations were identified ins/delins. NPM1 did not detect the translocation of AML 1-ETO and duplication FLT3-ITD. No chromosome aberrations were found.
19	46,XX,der(9)t(9;11;17)(q12;q23;q25),der(11)t(9;11)(p22?;q23),der(17)t(9;17)(q12;q23), del(17)(q23q25). FLT3 (-). KMT2A gene rearrangement on another chromosome was detected in 70% of the analyzed cells.
20	No data
21	No data
22	FLT3(-), WT1(-) NPM1 (-), Hema-Vision 28 N (28 most common rearrangements were not found).
23	46,XX,t(9;11)(9pter~9p22::11q23~11q14.2::9p22~9q32::11q23~11qter;11pter~ 11q14.2::11q2311q23::9q32~9qter) (4)/46,XX (17)
24	t(9;11)(p22;q23)
25	No mitoses, FISH 10% of cells with the KMT2A rearrangement
26	No data
27	No data
28	No data
29	No data
30	No data
31	No data
32	t(8;21)(q22;q22)
33	t(8;21)(q22;q22)
34	Any abnormalities
35	Complex karyotype with clonal evolution
36	t(8;21)(q22;q22)
37	No data
38	30% KOM + t(8;21)(q22;q22)
39	t(8,21)(q22;q22)
40	All negative in AML
41	Abnormal male karyotype with clonal evolution; test performed using the KMT2A-specific probe showed the presence of *KMT2A* gene rearrangement in 93% of the interphase nuclei; metaphases in the sample confirmed the t(9;11)(p21;q23) translocation.
42	No data
43	No data

*(*genetic results from tumor tissue in patient 5).*

TABLE 3 | Methods of MS treatment in the study group.

Treatment method	N	%
Chemotherapy	43	100%
Radiotherapy	24	56%
CNS	15	35%
tumor area	7	16%
CNS and tumor area	2	5%
Surgery	6	14%
Radical	5	12%
Subtotal	1	2%
Allogeneic hematopoietic stem cell transplantation	9	21%

while awaiting alloHSCT (17, 19). The second patient, a boy with a relapse of acute bilineal leukemia: T-cell acute lymphoblastic leukemia (T-ALL)/AML and a relapse of AML with MS in the mediastinum, received the treatment according to the Interfant 06 regimen, followed by palliative radiotherapy (20).

In four patients (9.30%) diagnosed with a disease other than MS, a different treatment regimen was used at baseline. Following the accurate diagnosis, the treatment was modified and the following regimens were used:

- for the treatment of lymphoblastic lymphoma (Euro LB 2002) (16) – 1 patient
- for Langerhans cell histiocytosis (HLH 2004) (21) – 1 patient
- for bone sarcoma (VIDE chemotherapy) (22) – 1 patient
- for non-Hodgkin lymphoma (2 x COP prephase according to the Inter-B-NHL Ritux 2010 protocol) (23) – 1 patient

In the second-line treatment, the protocol for AML relapses (Ida-Fla, Fla – 9 patients) was applied most frequently (19), with one patient receiving a regimen with clofarabine (24). Three patients underwent alloHSCT, one of whom had had donor lymphocyte infusion (DLI).

2. Treatment Outcomes

In the study group, CR in time was achieved in twenty-nine patients (67%), LR in six patients (14%), and PR in two patients (5%). Five (12%) patients progressed and seven patients (16%) relapsed. Fourteen patients (32%) died due to progression (eleven patients), infectious complications (two patients), and treatment-related toxicity (one patient).

The results of treatment outcomes were as follows (mean ± SD): OS=121.00 ± 13.23 months, pOS=0.67 ± 0.08; RFS=148.09 ± 11.77, pRFS=0.79 ± 0.07; EFS=112.82 ± 13.52, pEFS=0.65 ± 0.08. **Figure 1** shows pOS, pRFS, and pEFS curves.

3. Examining the Influence of Selected Factors on Treatment Outcomes

Age Group

No differences were found between the age groups (<10 years and >10 years) in OS, RFS, or EFS. Longer OS, RFS, and EFS were observed in younger patients (<10 years), but the differences were not significant (p=0.3584, p=0.8613, and p=0.8613, respectively).

Clinical Presentation of MS

OS and EFS were significantly longer in patients with MS and concurrent bone marrow involvement by AML/MDS compared to patients with *de novo* MS (**OS**=156.94 ± 13.89 vs. 89.75 ± 19.09; **p=0.0251, EFS**=150.97 ± 14.34 vs. 64.36 ± 12.68; **p=0.0247**). Curves for OS and EFS probability are shown in **Figures 2** and **3**.

Location

There were no significant differences in OS, RFS, or EFS between patients with MS in the skin and those with MS in other sites. Patients with the skin presentation of MS had lower pRFS than those with MS involving other sites, although this difference was not significant (0.63 ± 0.16 vs. 0.87 ± 0.07, p=0.0981).

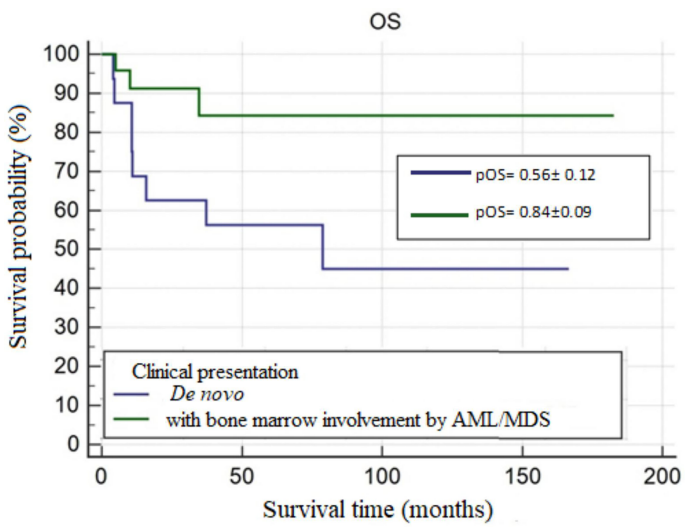

FIGURE 2 | The overall survival probability (pOS) in *de novo* MS and MS with bone marrow involvement by AML/MDS, p=0.0251.

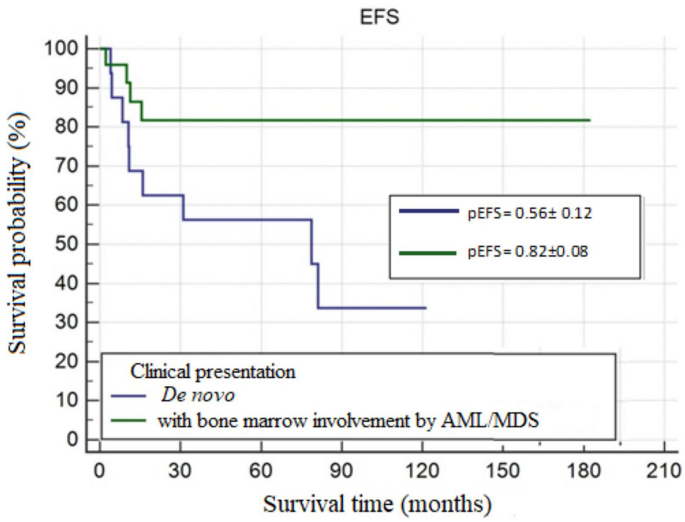

FIGURE 3 | The 5-year event-free survival probability (pEFS) by clinical presentation of MS — *de novo* and MS with bone marrow involvement by AML/MDS, p=.0247.

Compared to patients with MS in other sites, those with orbital MS were found to have shorter OS (96.91 ± 17.78 vs. 120.07 ± 15.08) and lower pRFS (0.74 ± 0.16 vs. 0.81 ± 0.08) and pEFS (0.64 ± 0.09 vs. 0.67 ± 0.16). However, the differences were not significant.

T(8;21)(q22;q22) Translocation

Patients with the t(8;21)(q22;q22) translocation had significantly longer EFS (67.65 ± 15.99 *vs.* 95.25 ± 8.39, **p=0.0490; Figure 4**) and were more likely to have longer OS (the latter not significant, p=0.0781) compared to those without the t(8;21)(q22;q22) translocation.

Treatment Method

The type of therapeutic protocol (AML-PPLLSG, AML-BFM 2004, AML-BFM 2012) did not significantly influence OS, RFS, or EFS.

Longer survival times and higher pOS, pRFS, and pEFS (without significance) were observed in patients who did not undergo alloHSCT.

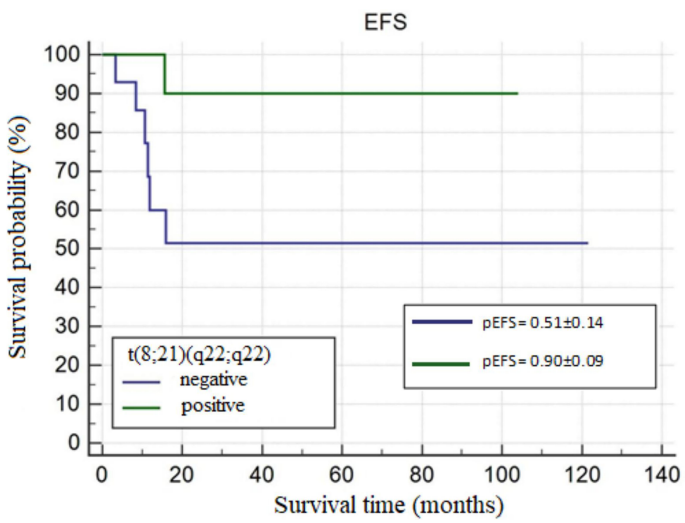

FIGURE 4 | The 5-year event-free survival probability (pEFS) in patients with and without t(8;21)(q22;q22), p=0.0490.

Neither surgical treatment nor radiotherapy significantly influenced treatment outcomes. Longer OS was found in patients who received radiotherapy (92.97 ± 19.32 vs. 53.88 ± 13.07).

4. Relapse Characteristics

Seven patients relapsed, four with a mixed relapse (medullary and extramedullary), two with an isolated extramedullary relapse, and one with an isolated medullary relapse. Out of a total of six patients with extramedullary relapse, five of them had extramedullary sites that were consistent with the primary location of MS. Primary clinical presentation with MS location, time from remission to relapse (months), and relapse location in patients who relapsed are presented in **Table 4**. The shortest time from MS diagnosis to relapse was 2.33 months, while the longest was 182.63 months (median, 31 months).

5. Causes of Death

The major cause of death was disease progression (11 patients). Two patients died due to infectious complications, one of whom also experienced treatment-related toxicity. In the group of patients who died, the mean time from diagnosis to death was 18.52 ± 20.24 months (range 3.27-78.67; median, 10.83 months).

DISCUSSION

Clinical Characteristics of the Study Group Versus Literature Data

Based on the nationwide database of fourty-three patients diagnosed with MS, the most common clinical presentation was MS accompanying AML, which supports previous literature (3, 5, 9, 10).

As in most reports (25, 26), MS diagnosis usually coincided with AML diagnosis among analyzed patients. There were no asymptomatic patients. However, MS accompanying AML may be underestimated. An analysis by Meyer et al. showed that MS was detected incidentally by imaging studies in 24.5% of patients (27). Stove et al. reported that among 315 children with AML, 39 (12%) were diagnosed with MS at baseline (25). Considering the above findings, it is particularly important that both oncologists and radiologists are aware of this issue.

Isolated MS (*de novo*) is thought to occur less frequently and affect about 1-2% of patients with AML (26). A literature search yielded only two reviews of pediatric patients with isolated MS. In the first one Reinhardt et al. reported 37 children diagnosed with isolated MS in a time frame of 13 years (28). The second study included 10 pediatric patients in Japan (29).

In one patient database, there were as many as 16 patients with *de novo* presentation. In four of them, further diagnostic assessment led to the identification of bone marrow involvement 1 to 2 months after diagnosis.

This study showed that the skin was the most frequent site of MS involvement. Similar findings regarding pediatric patients were reported in analyses available in the literature, including studies by Reinhardt et al. (28), Stove et al. (25), and Dusenbery et al. (11), which demonstrated that the second most common site was the orbital region. Studies including pediatric as well as adult patients revealed different results. The most frequent sites of involvement were lymph nodes (30, 31), followed by the paravertebral region, and then the skin (30).

In the study group, skin manifestation was observed significantly more often in younger patients (under 10 years of age). Based on the studies by Hurley et al., the most common skin lesions were pink or, in some cases, bluish papules (31, 32).

The second most frequent site of MS involvement (n=11, 25.58%) was the orbital region, which is considered a typical site of MS manifestation in children compared to adults, in whom the orbital region is much less frequently involved. Exophthalmos, reported in five patients (11.62%), is a typical and major symptom of orbital MS (33).

Apart from site-related symptoms, the most common symptoms included visible swelling around the lesion/tumor (18.6%), fatigue (18.6%), and pain (16.27%). Patients presented with a variety of symptoms and each patient was a separate and interesting case.

Summary of Diagnostic Aspects of MS in Children

The gold standard for diagnosis of MS is a tumor biopsy. The histopathological appearance of MS is non-specific and polymorphic; thus, it is vital that the immunohistochemical findings be evaluated in the diagnostic process (34). In this

TABLE 4 | Characteristics of MS relapses.

No	Primary MS location	Clinical presentation of MS	Time from remission to relapse (months)	Sites of extramedullary relapse	Bone marrow involvement in relapse [yes/no]
1	Skin	MS with bone marrow involvement (AML)	1 month	Skin	NO (*bone marrow involvement one month after detection of skin involvement)
2	Lung	Isolated extramedullary relapse of CML	15 months	Lung	NO
3	Paravertebral tumor	MS with bone marrow involvement (AML)	12 months	Absence	YES
4	Orbit	MS with bone marrow and CNS involvement (AML, relapse)	11 months	Orbit	YES
5	Abdominal cavity	*De novo*	2 months	Abdominal cavity	NO
6	Skin	MS with bone marrow involvement (AML)	6 months	Orbit and CNS	YES
7	Skin	MS with bone marrow involvement (AML)	10 months	Skin	YES

study, twenty-nine patients (67.44%) had tumor biopsy performed. There were patients with isolated MS as well as patients with concurrent bone marrow involvement. Consideration should be given to whether biopsy is necessary in all clinical presentations of MS. According to the literature, biopsy and histopathology should be performed unless the risks of the procedure outweigh its benefits (35).

Four patients (9.30%) were initially diagnosed with another cancer (bone sarcoma, non-Hodgkin lymphoma, and Langerhans cell histiocytosis). Based on the literature, the percentage of diagnostic errors ranges between 25% and 47%, and MS is most frequently misdiagnosed as non-Hodgkin lymphoma (28, 29). This supports the need for a broad panel of antibodies in immunohistochemistry as well as other diagnostic tools.

Flow cytometry provides rapid results of large arrays of antibodies, aiding in diagnosing MS. Therefore, an extensive antibody panel should be performed using fresh tumor tissue provided that it is possible to obtain enough material (36).

A special insight is necessary to diagnose rare *de novo* presentation of MS. Murthy et al. highlighted that a peripheral blood smear should be performed in all patients with exophthalmos and diagnostic evaluation should be expanded to include flow cytometry of bone marrow based on individual indications (33).

Following the case histories contained in the database, to confirm that a patient has isolated MS, the flow cytometry of bone marrow should be performed and, in cases of normal results of aspiration biopsy, trephine biopsy and genetic tests of bone marrow and tumor tissue should be carried out to detect the presence of chromosome rearrangements characteristic of AML. Paraffin blocks for retrospective genetic testing provides valuable insight for MS, phasing out the use fresh samples as a consequence of the time delay in histopathology results and insufficient samples (37). Such tests would be of particular importance in isolated MS and would allow an attempt to classify MS based on the cytogenetic and molecular abnormalities, which would further determine the choice of the most optimal treatment.

In all patients who had PET-CT (4 patients), the scans showed metabolically active sites. It is believed that PET-CT can be an even more sensitive imaging method for MS than computed tomography (25, 27, 38). Because of possible dissemination of MS, PET-CT seems to have additional diagnostic value by imaging all sites of disease (39). In addition, while monitoring treatment outcomes, PET-CT may serve as a more accurate tool for assessing remission, especially if alloHSCT is due to be performed later.

Treatment Outcomes and Analysis of Selected Prognostic Factors in Children With MS

This study investigated the influence of the following clinical factors on treatment outcomes: patient age (below and above 10 years), MS presentation, MS location, and the presence of t(8;21)(q22;q22) translocation.

Age

In this study, longer OS, RFS, and EFS, as well as higher 5-year probability of OS, RFS and EFS, were observed among younger patients (<10 years of age). However, the differences were not significant. In the literature, data on the influence of age on treatment outcomes in patients with MS are extremely limited. The studies by Pileri et al. and Al.-Khaateb et al. showed that age did not influence treatment outcomes (40, 41). Nevertheless, it is known that age is a powerful independent prognostic factor in AML: higher age implies worse prognosis. Lower pOS, accounting for only 50-60%, is observed in adolescents and young adults (AYA) group, i.e., patients aged between 15 and 39 years (42). Bearing in mind that MS usually accompanies AML, perhaps these relationships can be extrapolated to a group of patients with MS.

Clinical Presentation

A few studies indicated better treatment outcomes in patients with isolated MS (11, 43–45). This was linked to smaller tumor mass at baseline in those patients compared to patients with MS and bone marrow involvement. By contrast, this study showed that OS **was significantly longer** among patients with MS and **concurrent bone marrow involvement by AML/MDS** compared to patients with *de novo* MS. An analysis by Reinhardt et al. on 34 children with *de novo* MS also reported lower pOS in patients with isolated MS than in those with AML (0.44 ± 0.09 *vs.* 0.55 ± 0.02). Moreover, higher relapse rates were observed among children with isolated MS. Reinhardt et al. highlighted the influence of diagnosis delay on increased risk of relapse (46). However, Pileri et al. did not find any significant differences between the outcomes of patients with isolated MS and those with accompanying AML (40). Unfortunately, reports comparing treatment outcomes of pediatric patients with isolated MS *versus* those with concurrent AML are lacking. Comparisons of treatment outcomes were more often carried out between patients presenting AML without extramedullary involvement and those with AML accompanying MS. Interesting data were published by Pramanik et al., who showed that mean EFS and OS were higher in patients with AML and MS (median EFS was 21.0 months and median OS was 37.1 months) than in those with AML without extramedullary involvement (47).

To conclude, most of the literature considers that isolated MS is associated with poor prognosis. However, it has not been established whether extramedullary disease at AML diagnosis in children is an adverse prognostic factor. It is likely that more powerful factors, e.g., molecular features of cancer cells, determine prognosis.

Location

The orbital region and skin, the most common sites of MS involvement in children, have the opposite prognostic significance in the literature. According to the study by Johnston et al., orbital involvement was associated with higher OS compared to patients without extramedullary disease (11). Kobayashi et al. found that extramedullary disease not involving the skin was a favorable prognostic factor (9). In this study, there were no significant differences between OS, RFS, and EFS in

patients with MS regarding extramedullary site, including the orbital region. An example of an unfavorable orbital MS course is a patient who presented relapsed orbital MS with concurrent bone marrow involvement.

Manifestation of MS in the skin is considered to be more aggressive and displays a poor prognosis (6, 31, 32). In this study, no significant difference was found between OS of patients with MS of the skin and patients with MS involving other sites (p=0.7017). However, patients with MS of the skin had lower pRFS (p=0.0981). Three patients with skin involvement at baseline relapsed, also in the skin site. This proves that MS of the skin is associated with a high risk of relapse.

t(8;21)(q22;q22) Translocation

The t(8;21)(q22;q22) translocation is the most frequently reported cytogenetic abnormality in MS, especially in the pediatric population (5, 36, 48). This was also confirmed by the results of our study. It is known that the t(8;21)(q22;q22) translocation is associated with a favorable prognosis (49). However, data on its prognostic significance in patients with MS are inconclusive. Although Johnston et al. showed that patients with orbital MS had better outcomes, they did not find a relationship between these results and the t(8;21)(q22;q22) translocation (11). Felice et al. found that the t(8;21)(q22;q22) translocation did not worsen treatment outcomes in patients with MS (50). In contrast, Byrd et al. demonstrated that patients with MS and the t(8;21)(q22;q22) translocation experienced worse treatment outcomes, which was associated with more frequent involvement of the meningeal and paraspinal areas (51).

In this study, patients with the t(8;21)(q22;q22) translocation had significantly longer time and probability of EFS, which supports a favorable prognostic value of t(8;21)(q22;q22).

Treatment

According to the literature, MS treatment should always be based on systemic polychemotherapy (3, 12, 26). This also applies to patients with isolated MS or with MS after complete surgical resection.

Indeed, apart from patients with MS relapse, all the others from the study group received systemic chemotherapy based on the current treatment regimen for AML. The longest mean OS (146.93 ± 22.25) and the highest pOS (0.80 ± 0.13) were found in patients who were treated with the AML-PPLLSG 98 regimen.

There are neither standards for surgical management and radiotherapy nor indications for alloHSCT in patients with MS, while data published thus far on this subject are ambiguous. In this study, no algorithm for such treatment was found.

Furthermore, there was no significant influence of surgical treatment on OS, RFS, and EFS. However, it is difficult to draw far-reaching conclusions due to a small number of patients; surgery was a part of treatment in six patients (13.95%) from the study group. Patients who did not undergo surgical treatment had longer OS, RFS, and EFS. One patient diagnosed with isolated extramedullary CML relapse (Pancoast tumor) had radical surgery performed to remove the lesion. After 15 months, one patient relapsed in the primary site. Therefore, it is most likely that the key role of surgical treatment is to reduce the symptoms caused by the pressure exerted by the tumor on surrounding structures (12, 35).

Although OS, RFS, and EFS were longer in patients who received radiotherapy, there was also no significant influence of radiotherapy on treatment outcomes. This is in accordance with previous analyses (12, 44). However, the efficacy of radiotherapy with fractionated doses of 20-30 Gy as a part of palliative treatment has been proven in patients with MS (45, 49). Bakst et al. assessed the effects of radiotherapy in 22 patients with MS, indicating that radiotherapy was administered to 90% of the patients for relapse treatment or as a part of first-line treatment in the presence of a residual mass following chemotherapy. Improvement in local symptoms was observed in 95% of patients and treatment was well tolerated (49).

In this study, two patients who received radiotherapy as palliative treatment experienced pain reduction and improvement in symptoms resulting from pressure. The literature describes only single cases of patients in whom radiotherapy was an effective treatment of MS. Yang et al. reported the case of a 19-year-old patient diagnosed with isolated cardiac MS after three years from alloHSCT for AML. Following radiotherapy, the patient improved quickly and achieved remission lasting for six months. Authors put forward a hypothesis that patients post-alloHSCT especially benefit from the radiotherapy due to radiation-induced GvL (52).

Minoia et al. described the case of a 71-year-old woman with MS involving both breasts treated effectively with decitabine and radiotherapy with a dose of 30 Gy (53).

In contrast to some previous reports, no beneficial effect of allotransplantation on outcomes was found in this study (3, 54). Significantly longer RFS was observed in patients who did not undergo hematopoietic stem cell transplantation (p=0.0124). This may arise from the fact that patients with AML and a high risk of relapse and treatment failure are eligible for alloHSCT.

The first-line treatment included alloHSCT in three patients of those who relapsed. Several studies have revealed that the presence of extramedullary disease in patients before alloHSCT is one of the risk factors of extramedullary relapse. To what extent the GvL effect following alloHSCT is effective in extramedullary disease is subject to considerable debate (14, 15). Overall, further broad clinical studies should be done to investigate the role of alloHSCT in the treatment of MS as well as to search for effective methods of prevention and treatment of extramedullary relapse after alloHSCT.

In recent years, chimeric antigen receptor T-cell (CAR-T) therapy has become a revolution in the treatment of myeloproliferative disorders. This therapy uses genetically modified T-cells that acquire the ability to kill a cancer cell. Such type of immunotherapy is already used in the treatment of refractory acute lymphoblastic leukemia and refractory lymphoma. Its use in refractory AML and other myeloproliferative neoplasms is being studied in clinical trials (55). It is unknown how effective it would be in extramedullary disease.

Sorafenib, a kinase inhibitor, offers hope for the treatment of refractory solid tumors and refractory myeloid leukemia in children, especially with the presence of the FLT3-ITD mutation (56). To date, there have been no reports of the use of sorafenib in pediatric patients with MS. Grillo et al. presented a case of an adult patient with extramedullary relapse of AML as MS involving multiple sites after alloHSCT. The patient was successfully treated with sorafenib and subsequent DLI. Genetic tests of tumor tissue revealed the presence of the the FLT3-ITD mutation (56).

Drugs affecting apoptosis may also become a novel option for the treatment of refractory AML. Likewise, inhibitors of BCL-2 (venotoclax), which are already used in the treatment of refractory leukemia in adults, provide hope for improving treatment outcomes in children with refractory myeloid leukemia (57, 58).

SUMMARY

The evolving nature of revelations of MS warrants specific modern therapeutic regimens. As more and more reports of MS occur due to the enrichment and awareness of MS, it is vital to take all reports into account to examine as a whole the response to current and new treatments in order to prevent relapses, provide optimal treatment, and accurately assess prognosis.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The dataset was created by the author on the basis of clinical data and patient's history available in pediatric oncology centers. The author has access to the data which are not public. Requests to access these datasets should be directed to samborska.magda@gmail.com.

AUTHOR CONTRIBUTIONS

MS and KD designed the study. JW, ST revised critically the manuscript. MS, MB, JS-S, MC, WBal, SK, KP, MW, TO, TU, GW, JW-T, BU, AC, AK, MK-R, AS-B, IM, MM, AM-M, RT, TS, AC-G, GK, LM-K, NI-J, WBad, MD, PK collected the clinical data. MS drafted the manuscript. KD edited and revised the manuscript. All authors contributed to the article.

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BRIEF RESEARCH REPORT
published: 13 July 2022
doi: 10.3389/fonc.2022.932337

Molecular Heterogeneity in Pediatric Malignant Rhabdoid Tumors in Patients With Multi-Organ Involvement

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OPEN ACCESS

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Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

Received: 29 April 2022
Accepted: 09 June 2022
Published: 13 July 2022

Citation:
Miller KE, Wheeler G, LaHaye S, Schieffer KM, Cearlock S, Venkata LPR, Bravo AO, Grischow OE, Kelly BJ, White P, Pierson CR, Boué DR, Koo SC, Klawinski D, Ranalli MA, Shaikhouni A, Salloum R, Shatara M, Leonard JR, Wilson RK, Cottrell CE, Mardis ER and Koboldt DC (2022) Molecular Heterogeneity in Pediatric Malignant Rhabdoid Tumors in Patients With Multi-Organ Involvement. Front. Oncol. 12:932337. doi: 10.3389/fonc.2022.932337

Rhabdoid tumors (RTs) of the brain (atypical teratoid/rhabdoid tumor; AT/RT) and extracranial sites (most often the kidney; RTK) are malignant tumors predominantly occurring in children, frequently those with *SMARCB1* germline alterations. Here we present data from seven RTs from three pediatric patients who all had multi-organ involvement. The tumors were analyzed using a multimodal molecular approach, which included exome sequencing of tumor and germline comparator and RNA sequencing and DNA array-based methylation profiling of tumors. *SMARCB1* germline alterations were identified in all patients and in all tumors. We observed a second hit in *SMARCB1 via* chr22 loss of heterozygosity. By methylation profiling, all tumors were classified as rhabdoid tumors with a corresponding subclassification within the MYC, TYR, or SHH AT/RT subgroups. Using RNA-seq gene expression clustering, we recapitulated the classification of known AT/RT subgroups. Synchronous brain and kidney tumors from the same patient showed different patterns of either copy number variants, single-nucleotide variants, and/or genome-wide DNA methylation, suggestive of non-clonal origin. Furthermore, we demonstrated that a lung and abdominal metastasis from two patients shared overlapping molecular features with the patient's primary kidney tumor, indicating the likely origin of the metastasis. In addition to the *SMARCB1* events, we

identified other whole-chromosome events and single-nucleotide variants in tumors, but none were found to be prognostic, diagnostic, or offer therapeutic potential for rhabdoid tumors. While our findings are of biological interest, there may also be clinical value in comprehensive molecular profiling in patients with multiple rhabdoid tumors, particularly given the potential prognostic and therapeutic implications for different rhabdoid tumor subgroups demonstrated in recent clinical trials and other large cohort studies.

Keywords: atypical teratoid/rhabdoid tumor (AT/RT), malignant rhabdoid tumor (MRT), SMARCB1, next-generation sequencing, DNA methylation array

INTRODUCTION

Rhabdoid tumors (RT) are rare, malignant tumors diagnosed most often in early childhood. RTs are classified according to their anatomical location and most often arise in the brain (atypical teratoid/rhabdoid tumor; AT/RT) and/or extracranially, usually in the kidney (RTK) and sometimes in other soft tissues like muscles (1, 2). In the United States, AT/RT represents 10% of primary brain and central nervous system (CNS) tumors diagnosed in individuals less than 1 year of age (1). RTKs account for 18% of all renal tumors diagnosed in infants (2). Because of the rarity and aggressiveness of RTs and given the young age of many patients, there is no defined standard of care, and RTs remain one of the most lethal childhood tumors with overall survival rates <50% (3–5).

Almost all cases of RT, regardless of anatomical site, are molecularly characterized by the biallelic alteration of *SMARCB1*, leading to complete inactivation of the gene and, more rarely, inactivation of *SMARCA4* in a germline or somatic setting (6–8). *SMARCB1*, also called *INI1/BAF47/SNF5*, is an established tumor suppressor at 22q11.2 that encodes a subunit protein of the SWI/SNF chromatin remodeling complex (9). In approximately 35% of individuals diagnosed with an AT/RT or RTK, one of the *SMARCB1* alterations is present in the germline, predisposing the individual to the development of rhabdoid tumors (10, 11).

Despite the commonality of being driven by *SMARCB1* or *SMARCA4* loss, there is molecular and clinical heterogeneity among AT/RT. There are three known DNA methylation subgroups associated with AT/RT, referred to as TYR (characterized by the overexpression of melanosomal genes), SHH (characterized by the overexpression of sonic hedgehog signaling pathways), and MYC (characterized by the overexpression of both MYC proto-oncogene and HOX cluster genes) (12–14). AT/RT-TYR are diagnosed in the youngest population group (median age of diagnosis: 12 months) and usually arise in an infratentorial location (75%). AT/RT-SHH are predominantly supratentorial in location (65%) and are diagnosed in individuals with a median age of 20 months. AT/RT-MYC arise in the supratentorial region (50%), the infratentorial region (38%), and even extracranially in the spinal cord (12%) and represent the oldest population group within AT/RT diagnoses at a median age of 27 months. Extracranial rhabdoid tumors most often show a similarity with the AT/RT-MYC subgroup at the DNA methylation level (15, 16). The commonalities and overlap between subgroups of AT/RT and subgroups of extracranial RT (specifically, RTK) are not well characterized, particularly in patients who present with both types of tumors or in longitudinal samples from patients who experience metastasis of their primary tumor.

Here we present a case series of three pediatric patients diagnosed and treated for AT/RT and/or RTK. We analyzed multiple different tumors from each patient, totaling seven tumor samples, including primary tumors and metastases. Each tumor was comprehensively analyzed using a multimodal molecular characterization approach that included exome sequencing for detection of germline and somatic variants and copy number alterations, whole transcriptome sequencing (RNA-seq), DNA array-based methylation analyses, and clonality analysis. Our approach uncovered a consistent biallelic inactivation of *SMARCB1* as expected but revealed molecular heterogeneity among tumors from the same individual.

METHODS

Human Subjects

Written informed consent was obtained for all participants in this study under a research protocol approved by the Institutional Review Board at Nationwide Children's Hospital (IRB17-00206). We enrolled three individuals with a diagnosis of rhabdoid tumor. Our report includes three females: "patient 1", "patient 2", and "patient 3" diagnosed at age 10, 2, and 2 months, respectively. Two patients presented with multifocal synchronous AT/RT and RTK primary tumors, one of whom eventually had metastasis to the lungs. The third patient had a primary RTK and ultimately experienced tumor metastasis to the abdomen after an initial surgical resection. Snap-frozen disease-involved tissue was studied, when possible, but for some specimens only formalin-fixed, paraffin-embedded (FFPE) disease-involved tissue was available for study. A detailed description of the clinical history of each patient can be found in the supplementary file (**Case Descriptions**).

Samples and Extractions

Normal comparator tissue was obtained from blood-derived peripheral blood mononuclear cells or from non-tumor kidney tissue in one individual. The tumor samples were obtained as either fresh-frozen or FFPE tissue and were used for the co-extraction of DNA (AllPrep DNA kit, Qiagen) and RNA (mirVana isolation kit, Thermo Fisher Scientific for frozen and High Pure isolation kit, Roche Life Science for FFPE tissues).

Exome Sequencing and Analysis

Sequencing libraries were prepared for exome sequencing using NEBNext Ultra II FS DNA library prep kit (New England BioLabs). First, target enrichment by hybrid capture was performed by combining xGen Exome Research Panel with the xGenCNV Backbone and Cancer-Enriched Panels-Tech Access (Integrated DNA Technologies). The xGen exome panel (catalog number 10005153) targets 19,433 genes with a total probe coverage encompassing 39 Mb of genomic space, while the xGenCNV backbone panel (catalog number 1080569) consists of 9,115 individually synthesized probes combined with an additional 1,855 probes enriching cancer-associated gene regions. Libraries were then generated using the NEBNext Ultra II FS kit, and paired-end 151-bp reads were generated on NovaSeq6000. Alignment to human reference genome build GRCh38 and secondary analysis were performed using our previously published pipeline (17).

Germline variants were called using GATK's HaplotypeCaller. The variants were then filtered based on the following characteristics: gnomAD population frequency <0.0001, depth of sequencing ≥8 reads, variant within protein-coding region or within 3 base pairs of canonical splice site, and presence of the gene within a previously published cancer predisposition list of 565 genes (18). VarScan2 and GATK were used to assess copy number variants (CNVs) and to detect loss of heterozygosity across all chromosomes (19). Copy number variation plots were generated from Varscan2-called segment files and plotted by centering around a zero-point determined by the median log-2 value of the segments of the first five chromosomes. Invariant copy data based on log-2 ratios were plotted as 100-bp windows in blue, wherein copy number variant segments were plotted as a red line by corresponding log-2 copy ratio and position. Loss of heterozygosity (LOH) data were plotted for tumor samples using the position (X axis) and variant allele frequency (VAF; Y axis) of called alleles filtered by a list of known biallelic sites. Points falling outside of an expected normal range of 25 to 75% were marked as LOH-supporting.

Somatic variants were called using MuTect2 (20). Somatic nonsynonymous SNVs and small insertions or deletions (indels) were filtered for quality (site quality ≥100), population frequency (gnomAD population frequency <0.0001), absence in the germline comparator sample, somatic alternate allele read depth (≥4 reads), minimum tumor VAF ≥5%, and gene location within a coding or splice site (≤3 base pairs) region. Variants passing all the aforementioned filters were manually reviewed in Integrated Genomics Viewer and then analyzed for the presence of the variant within a previously defined cancer hotspot (21) or the presence of the gene within a previously published cancer predisposition list of 565 genes (18). VarScan2 and GATK were used to assess CNVs and loss of heterozygosity across all chromosomes (19). Copy number variation and LOH data plots were generated as described for germline CNVs.

DNA Array-Based Methylation Profiling

For each tumor studied, 250–500 ng of input DNA was bisulfite-converted (catalog number D5006, Zymo Research, Irvine, CA, USA) and, if applicable, treated using the Illumina FFPE restoration process (catalog number WG-321-1002, Illumina, San Diego, CA, USA). Bisulfite-converted DNAs, including methylated human DNA controls (catalog number D5014, Zymo Research, Irving, CA, USA), were hybridized to the Infinium Methylation EPIC BeadChip (catalog number WG-317-1001, Illumina, San Diego, CA, USA) following the Illumina Infinium HD Methylation protocol. Beadchips were imaged on the Illumina iScan System, and the resulting raw IDAT files were processed through a local installation of the German Cancer Research Center (DKFZ) DNA Methylation Brain Tumor Classifier, version 11b4 or 11b6 (22, 23). Uniform manifold approximation and projection (UMAP) plots were generated to assess the unsupervised clustering of the studied AT/RT samples, where only the most differentially methylated probes were considered. For comparison of our study samples with external DKFZ embryonal tumor samples (*i.e.*, AT/RT, medulloblastomas, and embryonal tumors with multilayered rosettes), standard deviation ≥0.25 was used, which included 30,549 most differentially methylated probes for clustering analyses.

RNA Sequencing and Gene Expression Analysis

Tumor RNA was subjected to DNase treatment and ribodepletion prior to library construction using NEBNext Ultra II Directional RNA library prep kit for Illumina (New England BioLabs). Paired-end 151-bp reads were generated on Illumina HiSeq 4000 and aligned to the human genome reference sequence build GRCh38. Alignment was performed using a custom in-house pipeline and the splice-aware aligner STAR (24). Clustering was performed by principal component analysis (PCA) on $\log10(x + 1)$ and quantile-normalized DESeq2 expression values using a panel of 36 genes with known relevance to AT/RT subtyping for MYC (*HOTAIR*, *HOXC4/5/6/8/9/10/11/12/13/AS1/AS5*, and *MYC*), SHH (*ASCL1*, *BOC*, *CDH6*, *DLL1/3*, *DTX1*, *GLI2*, *HES1/5/6*, *MYCN*, and *PTCH1*), and TYR (*BMP4*, *DCT*, *DNAH11*, *FGFR2*, *JAK1*, *MITF*, *OTX2*, *PDGFRB*, *SPEF1*, *TYR*, and *VEGFA*) groups (13).

Clonality Analysis

We used superFreq with default parameters to determine clonality (25). superFreq uses exome BAM files from tumor samples and identifies tumor-specific single-nucleotide variants, indels, and copy number variants to track clones across multiple samples from the same patient.

RESULTS

Tissue Pathology

The pathology review typically estimated a high tumor cellularity/content (average, 95%; range, 90–100%) and a wide range of necrosis (average, 12%; range, 0–40%) for all tumors studied (**Table 1**). As described in the clinical summaries (**Supplementary File- Case Descriptions**), the histologic findings in all tumors were determined by board-certified

pathologists and were consistent with either AT/RT or extracranial RT supported by loss of INI1 staining.

Genomic Analysis

The average exome sequencing coverage depth for the tumor samples was 241X (range: 181X–289X) and for the germline comparator samples was 216X (range: 186X–266X). For all samples, an average of 97.9% of coding bases was covered by at least 20 reads (range, 97.0–98.6%) (**Supplementary Table S1**).

Germline Analysis

All three patients had a pathogenic germline alteration affecting *SMARCB1* identified by exome sequencing (**Table 1**). Patient 1 had a heterozygous frameshift variant in *SMARCB1* (p.Pro215Leufs*14), while patients 2 and 3 had a large deletion (>1 Mb) of chr22q11, a region which includes the *SMARCB1* gene, present in germline comparator tissue, thus confirming a diagnosis of rhabdoid tumor predisposition syndrome for all three.

Somatic Analysis

We observed a second somatic hit in *SMARCB1* in all tumor specimens *via* LOH of chr22, inclusive of the *SMARCB1* gene region. In patient 1, although heterozygous in the germline (48% VAF), the p.Pro215Leufs*14 variant exhibited much higher allele frequencies in all three tumor specimens (87% in primary brain, 68% in primary kidney, and 82% in lung metastasis) because of extensive copy-neutral LOH across chromosome 22q in all three tumor specimens (**Supplementary Figures S1A–C**). In patient 2, the chr22 deletion appeared homozygous in the patient's primary brain and primary kidney tumors due to copy-neutral LOH across the entirety of chromosome 22 (**Supplementary Figures S1D, E**). We made similar observations in patient 3, who harbored a slightly smaller germline deletion on chr22 (1.34 Mb) that appeared homozygous in both primary kidney and metastatic abdominal tumors due to copy-neutral LOH affecting the entire chromosome (**Supplementary Figures S1F, G**).

DNA Methylation Profiling and Gene Expression Clustering and CNV Analysis Reveal Heterogeneity Between Tumors From the Same Patient

We generated DNA methylation profiles using Illumina EPIC 850K microarray for our cohort of tumors and analyzed the data using the DKFZ brain tumor methylation classifier v11b4 or v11b6, which provides the classification of 184 known central nervous system tumors (22). All tumors, including intra- and extracranial tumors, matched most closely with the methylation family of AT/RT, and all had confidence scores >0.90 (range: 0.9421–0.9997; **Table 1**). The brain tumor methylation classifier also assigns samples to different methylation subgroups or classes within the AT/RT family. In our cohort, one brain sample was predicted to be a TYR subgroup and one was predicted to be a SHH subgroup, while the other five samples (all non-CNS) were predicted to be MYC subgroups. Synchronous tumors from the same patient (patients 1 and 2) demonstrated epigenetic heterogeneity and were assigned different methylation subgroups, as visualized by the appearance in distinct UMAP clusters alongside other embryonal tumors from the DKFZ database (**Supplementary Figure S2**). The primary RTK and abdominal metastatic tumors from patient 3 were both classified as MYC and clustered together.

We also performed RNA-seq on all tumors, yielding >80,000,000 total reads for each RNA sample (**Supplementary Table S1**). To explore the heterogeneity of gene expression between the subgroups of RTs, we performed PCA of the RNA-seq data using 36 genes (see "*Methods*") known to be divergently expressed in specific AT/RT subgroups, which include TYR/MITF/others for TYR subgroup, NOTCH signaling genes (*e.g.*, *ASCL1*, *HES5/6*, and *DLL1/3*) and SHH signaling genes (*e.g.*, *MYCN* and *GLI2*) for SHH subgroup, and *MYC/HOTAIR/HOXC* cluster genes for MYC subgroup (13). Similar to our methylation clustering, synchronous brain and extracranial tumors from the same patient demonstrated a variation in expression profiles and appeared in distinct clusters (**Supplementary Figure S3**). The resultant gene

TABLE 1 | Summary of molecular findings.

ID	Comparatortissue	Germline*SMARCB1*	Tumorsite	Tumorcontent	Necrosis	CNS family (methylation score)	CNS AT/RT class (methylation score)
Patient 1	Blood	p.Pro215Leufs*14	Brain	95%	1%	AT/RT (0.9997)[a]	TYR (0.9997)[a]
			Kidney	90%	30%	AT/RT (0.9421)[a]	MYC (0.9078)[a]
			Lung (metastatic)	90%	40%	AT/RT (0.9998)[b]	MYC (0.9977)[b]
Patient 2	Kidney, non-tumor	1.88 Mb deletion at 22q11.22-q11.23	Brain	100%	0%	AT/RT (0.9980)[a]	SHH (0.9958)[a]
			Kidney	98%	5%	AT/RT (0.9971)[a]	MYC (0.9967)[a]
Patient 3	Blood	1.34 Mb deletion at 22q11.22-q11.23	Kidney	100%	0%	AT/RT (0.9997)[a]	MYC (0.9997)[a]
			Abdomen (metastatic)	90%	10%	AT/RT (0.9932)[b]	MYC (0.9823)[b]

Unless otherwise indicated, all tissue specimens were from a primary tumor. The estimates of tumor content and necrosis are based on a pathology review.
AT/RT, atypical teratoid rhabdoid tumor; MYC, MYC gene subgroup of AT/RT; SHH, sonic hedgehog subgroup of AT/RT; TYR, tyrosinase subgroup of AT/RT.
[a]*Classifier versions used for the Heidelberg Brain Tumor and Sarcoma Classifiers: v11b4 CNS classifier.*
[b]*Classifier versions used for the Heidelberg Brain Tumor and Sarcoma Classifiers: v11b6 CNS classifier.*

expression clustering data supported the subgroup classification predicted by methylation analysis for each sample.

Clonal Tracking Using Single-Nucleotide Variants and Copy Number Variations Reveals Heterogeneity Between Tumors From the Same Patient

Patient 1

While all samples possess second-hit somatic events resulting in the loss of any functional *SMARCB1* allele, the brain lesion demonstrated a whole-chromosome loss of chr22, whereas the two extracranial samples exhibited LOH of chr22q only. Clonality analysis differentiated the brain and extracranial lesions into two distinct tumorigenic origins on the basis of a number of other variants. Of the 25 SNVs included in clonal clustering, none was found to be shared between the brain and extracranial tumors (**Figure 1** and **Supplementary Table S2**). The kidney and lung tumors were found to be closely related, with the kidney and lung tumors sharing four SNVs. Like the patient's kidney tumor, the lung metastasis was also classified as MYC subgroup based on methylation profiling, further supporting the probable shared clonal origin of these two extracranial tumors. Each extracranial sample was found to possess a uniquely derived subclone, with kidney and lung samples gaining a number of exclusive SNVs (7 and 12, respectively). The subclone identified in the brain sample was differentiated only by one SNV, an intronic variant in *GPKOW* (**Supplementary Table S2**). No other CNVs besides chr22 or chr22q LOH were identified in any of the tumors (**Supplementary Figures S1A–C**). Despite the presence of two independent tumor lineages and as many as five distinct clones, no new diagnostic, prognostic, or therapeutically applicable SNVs or CNVs were found in any clones beyond the *SMARCB1* events.

Patient 2

Clonality analysis indicated a shared origin for kidney and brain samples as the most parsimonious clonal history for this case. However, only a single event—whole-chromosome copy-neutral LOH of chr22—is shared between the two tumors. While the kidney lesion was not found to have any SNVs or CNVs beyond the initial chr22 LOH, the copy number analysis of the brain tumor revealed several chromosomal aberrations and two distinct sequentially derived clones (**Figure 2**). The first clone

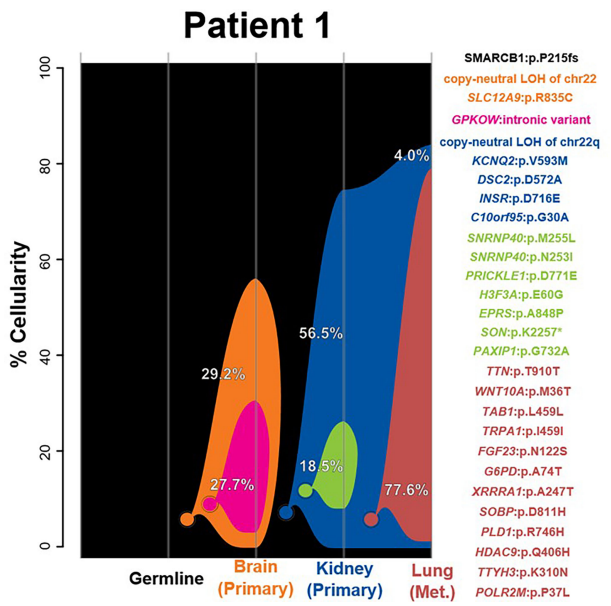

FIGURE 1 | **–3**Clonality analysis. River plots showing the composition of clones in multiple tumor samples plus matched normal blood (germline) sample. Vertical lines indicate a sample, labeled on the X-axis. Colored circles indicate the origins of a clone from a single cell, either of the germline (black) or a preceding clone. Colored outlines show the cellularity of clones (Y-axis) in each sample. The percentages shown (in white or black text) indicate the cellularity of a tumor clone in each sample that was identified, excluding the cells of any descendant clones. Clone percentages sum up to the total tumor cellularity of a sample. The samples are arranged to most clearly visualize clonal descent but do not represent a formal time-series. The copy number variants, loss-of-heterozygosity events, and single-nucleotide variants identified in the tumor samples as well as relevant germline predisposition variants are listed on the right and colored by which clone they belong to.

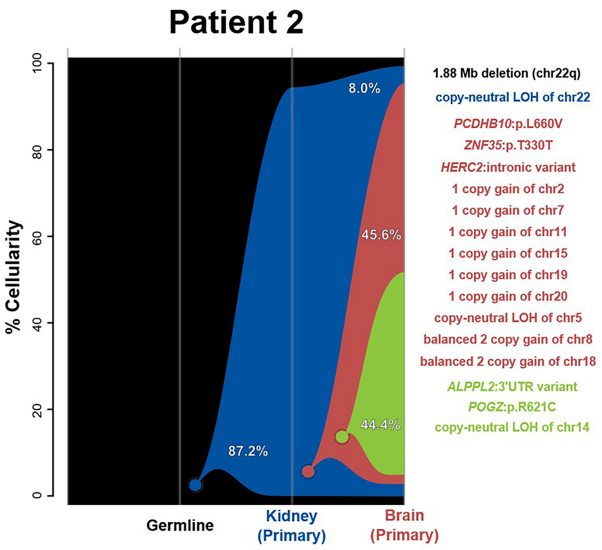

FIGURE 2 | River plots show the composition of clones in multiple tumor samples plus matched normal blood (germline) sample. Vertical lines indicate a sample, labeled on the X-axis. Colored circles indicate the origins of a clone from a single cell, either of the germline (black) or a preceding clone. Colored outlines show the cellularity of clones (Y-axis) in each sample. Percentages shown (in white or black text) indicate the cellularity of a tumor clone in each sample that was identified, excluding the cells of any descendant clones. Clone percentages sum to the total tumor cellularity of a sample. Samples are arranged to most clearly visualize clonal descent but do not represent a formal time-series. Copy number variants (CNVs), loss-of-heterozygosity events (LOH), and single nucleotide variants (SNVs) identified in the tumor samples, as well as relevant germline predisposition variants, are listed on the right and colored by which clone they belong to.

(red) added three SNVs and nine full-chromosome CNV events —a single-copy gain of chr2, chr7, chr11, chr15, chr19, and chr20; balanced two-copy gains of chr8 and chr18; and a copy-neutral LOH of chr5 (**Figure 2** and **Supplementary Figures 1D, E**). The second, further-derived clone (green) added an additional of two SNVs plus a loss of heterozygosity of chr14. These observed molecular differences between the brain and kidney tumors is further supported by their classification into different methylation subgroups (SHH and MYC, respectively), indicating that the tumors do not share a clonal origin. Aside from chr22 LOH, none of the SNVs or CNVs unique to the brain lesion was found to be meaningful in terms of prognosis, diagnosis, or therapeutic potential for AT/RT (26).

Patient 3

The two samples in this case, of the kidney and abdomen, were determined to share a single tumorigenic origin on the basis of shared SNVs in *MST1* and *LINGO4*, in addition to the chr22 LOH event often observed in rhabdoid tumors (**Figure 3** and **Supplementary Figures 1F, G**). Methylation profiling classified both as MYC, supporting the abdominal metastases that share similar epigenetic features to the kidney tumor and therefore may share a clonal origin. From this shared clonal origin, three additional distinct subclones have arisen. The one subclone present in the kidney (red) sample possesses five additional SNVs, while the two sequentially derived abdominal subclones add 49 (green) and 9 (orange) SNVs, respectively. In each sample, the derived clones have nearly entirely replaced the ancestral cell population, resulting in the two tumors being substantially genetically distinct in a large number of high-frequency variants; none of these derived variants, however, was found to be informative of prognosis, diagnosis, or therapeutic potential for rhabdoid tumors.

DISCUSSION

We analyzed seven tumor tissue samples from three pediatric patients diagnosed with rhabdoid tumors of the brain and/or extracranial sites and aimed to systematically assess each individual case using a multimodal approach of DNA and RNA sequencing plus methylation profiling. As expected, all tumors exhibited biallelic inactivation of *SMARCB1*, including a germline *SMARCB1* alteration in every patient. We also analyzed tumors using DNA array-based methylation and RNAseq-based gene expression analyses to characterize tumors based on known molecular subgroups of AT/RT (12). Our use of the v11b4/b6 brain tumor methylation classifier provided confident classifications for tumors as either MYC, SHH, or TYR AT/RT subgroups, even for extracranial RTs (22). We confirmed the use of analyzing gene expression data from RNA-seq as an orthogonal method for identifying distinct molecular AT/RT subgroups by evaluating the expression of known subgroup-specific marker genes (13).

Our integrated analysis of synchronous tumors from the same patient revealed several interesting findings. First, RTs originating in and outside the brain in each case showed molecularly heterogeneous methylation profiling and were classified as different subgroups (patient 1 brain = TYR and kidney = MYC; patient 2 brain = SHH and kidney = MYC). While the finding of divergent methylation patterns in synchronous rhabdoid tumors has been reported before, our integrated analyses using exome

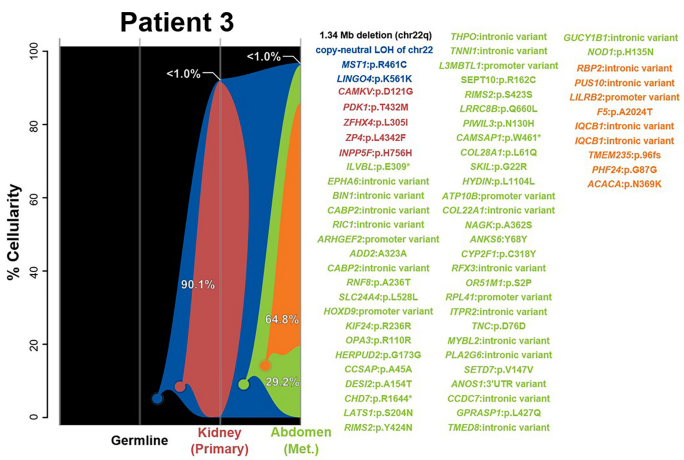

FIGURE 3 | River plots show the composition of clones in multiple tumor samples plus matched normal blood (germline) sample. Vertical lines indicate a sample, labeled on the X-axis. Colored circles indicate the origins of a clone from a single cell, either of the germline (black) or a preceding clone. Colored outlines show the cellularity of clones (Y-axis) in each sample. Percentages shown (in white or black text) indicate the cellularity of a tumor clone in each sample that was identified, excluding the cells of any descendant clones. Clone percentages sum to the total tumor cellularity of a sample. Samples are arranged to most clearly visualize clonal descent but do not represent a formal time-series. Copy number variants (CNVs), loss-of-heterozygosity events (LOH), and single nucleotide variants (SNVs) identified in the tumor samples, as well as relevant germline predisposition variants, are listed on the right and colored by which clone they belong to.

sequencing and clonal tracking demonstrated that, aside from chr22 LOH, the brain and extracranial tumors demonstrated further molecular heterogeneity and never shared any tumor SNVs or CNVs (**Figures 1**, **2**) (15, 27). Although the tumors from patient 2 were initially predicted to share a clonal origin (using superFreq software), this is likely because of the commonality of chr22 LOH and the fact that the kidney tumor did not possess any other CNVs or SNVs. In fact, LOH of chr22 or chr22q was a ubiquitous event observed in all tumors within our cohort and is likely the most common mechanism for a second hit in *SMARCB1* in patients with rhabdoid tumor predisposition syndrome (*i.e.*, germline *SMARCB1* mutation). As chr22 LOH is common in this tumor type, it is most likely that the true explanation is that an indistinguishable event occurred in two separate instances for the brain and extracranial RT to have been formed—for example, in patient 2. This explanation is supported by the high cellularity of the first derived brain clone (red) in patient 2, suggesting that it may be the true tumorigenic ancestor of the brain lesion (**Figure 2**). Due to the lack of any other shared genomic alterations (besides chr22 LOH) and the lack of a biological mechanism for metastasis between the kidney and brain, the more plausible explanation is that the loss of chr22 has occurred twice separately. However, as the events alone are indistinguishable, a metastatic event is conceivably possible and cannot be excluded with certainty. In patient 3, we observed the same methylation profile for both kidney and abdominal lesions, and additionally we identified two shared SNVs between the two tumors at >40% VAF, indicating a likely shared clonal origin (**Supplementary Table S2**). While most of the tumors in our cohort appeared to derive clones, it was composed of either non-coding SNVs (intronic or promoter regions) or other passenger mutations, primarily nonsynonymous variants in genes not previously associated with cancer. Interestingly, only one tumor in our cohort (patient 2 brain) had additional CNVs besides chr22 LOH, highlighting the importance of using exome sequencing to study the tumors fully.

Given that AT/RT is a heterogenous disease with different subgroups, the goal is that molecular studies and findings can be used to guide therapy and improve patient outcomes. Several epidemiological studies have independently reported that patients with AT/RT-TYR or *ASCL1*-expressing (NOTCH signaling) ATRT-SHH tumors have a better prognosis, but more analyses on larger prospective cohorts are needed to confirm these findings (12, 28–30). In addition, several preclinical studies have identified drugs and drug-like inhibitors with different therapeutic effects in molecular subgroups of AT/RTs (31–33). Therefore, it is likely that molecular subgrouping of rhabdoid tumors is expected to affect patient management in the future, as there may be differences in response to different therapies and overall survival. While our findings are of biological interest, there may also be clinical value in using comprehensive molecular profiling to diagnose and classify rhabdoid synchronous tumors, particularly given the potential prognostic and therapeutic implications for different rhabdoid tumor subgroups.

DATA AVAILABILITY STATEMENT

DNA and RNA sequencing data for this study has been deposited to dbGaP under accession number phs001820.v1.p1. dbGaP identifiers for each sample are listed in **Supplementary Table 1**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board at Nationwide Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor (s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

KM: Conceptualization, Writing- Original Draft; GW: Formal analysis, Writing- Original Draft; SL: Formal analysis; KS: Formal analysis, Writing - Review and Editing; SC: Formal analysis, Writing- Original Draft; LV: Formal analysis; AB: Formal analysis; OG: Formal analysis; BK: Formal analysis; PW: Supervision; CP: Resources; DB: Resources, Writing - Review and Editing; SK: Resources; DK: Resources; MR: Resources; AS: Resources; RS: Resources; MS: Resources; JL: Resources; RW: Supervision; CC: Supervision, Writing - Review and Editing; EM: Conceptualization, Writing- Original Draft; DCK: Conceptualization, Writing- Original Draft. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Nationwide Foundation Pediatric Innovation Fund.

ACKNOWLEDGMENTS

We are grateful for the patients and families who participated in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.932337/full#supplementary-material

Supplementary Figure 1 | Genome-wide copy number plots. In the top window of each panel are copy number plots, where blue points represent the log-2 ratio for

the tumor relative to the normal specimen and red lines represent copy number variants segments as called by GATK. The heterozygosity plots (bottom window of each panel) show the tumor variant allele frequency (VAF) for heterozygous germline variants, which are colored red if they exhibit significant evidence of loss of heterozygosity (LOH) from the expected 50% VAF. The horizontal blue lines indicate contiguous LOH segments. Included are patient 1 primary brain tumor **(A)**, primary kidney tumor **(B)**, and metastatic lung tumor **(C)**; patient 2 primary brain tumor **(D)**, and primary kidney tumor **(E)**; and patient 3 primary kidney tumor **(F)** and metastatic abdominal tumor **(G)**.

Supplementary Figure 2 | Unsupervised clustering by uniform manifold approximation and projection (UMAP) of embryonal tumors indicates the grouping of rhabdoid tumor (RT) tumors by methylation classification. The seven samples from our study were compared to 545 embryonal tumors [atypical teratoid (AT)/RT, medulloblastoma, and embryonal tumors with multilayered rosettes] described by Capper *et al.* (22) by unsupervised UMAP clustering, using the most differentially methylated probes (standard deviation ≥0.25, n = 30549 probes). The seven tumors from our cohort were grouped according to their predicted classifications, as assigned by the DKFZ CNS Classifier v11b4/v11b6. The samples from our cohort are outlined in black and are filled with color by their AT/RT subgroup as called by previous methylation profiling.

Supplementary Figure 3 | Clustering by RNA-seq expression recapitulates subgrouping of rhabdoid tumors. Principal component analysis was performed by utilizing 36 genes (see "*Methods*") known to be differentially expressed in distinct AT/RT subgroups. The samples are colored by their RT subgroup as called by methylation profiling and as central nervous system (CNS) or non-CNS indicative of the tumor location.

Supplementary Table 1 | Exome sequencing metrics. Top: the 10 samples obtained from individuals are listed along with the type of tissue (tumor or comparator), sample type, mean depth of target exon coverage, and percent of targeted bases per gene sequenced to 20× coverage. Bottom: metrics from RNA-sequencing. The seven samples upon which RNA-seq was performed are listed along with tumor type, sample preservation type, total number of reads, number of reads mapped to GRCh38, and number of coding and untranslated region reads. The corresponding dbGap ID for each sample is listed. FFPE, formalin-fixed paraffin-embedded; GRCh38, human genome assembly (hg38) from Genome Reference Consortium; UTR, untranslated region

Supplementary Table 2 | Single-nucleotide variants and copy number variants detected in clonality analysis. Variants detected in clonal tracking are listed with corresponding variant allele frequencies predicted from exome sequencing data. The color of the clone corresponding to –**5** is also noted.

CASE DESCRIPTIONS | Clinical history of all three patients, which includes diagnostic details, treatment regimens, and the current clinical status of each patient.

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frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 05 September 2022
DOI 10.3389/fonc.2022.946281

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 17 May 2022
ACCEPTED 08 August 2022
PUBLISHED 05 September 2022

CITATION
Otth M, Denzler S, Diesch-Furlanetto T and Scheinemann K (2022) Cancer knowledge and health-consciousness in childhood cancer survivors following transition into adult care—results from the ACCS project. *Front. Oncol.* 12:946281. doi: 10.3389/fonc.2022.946281

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Cancer knowledge and health-consciousness in childhood cancer survivors following transition into adult care—results from the ACCS project

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Background: Knowledge on chronic medical conditions in childhood cancer survivors (CCSs) is constantly growing and underlines that long-term follow-up (LTFU) care is often mandatory, also in adulthood. However, many CCSs discontinue follow-up care after transition to adult care. One reason might be that the current transition practices do not meet the needs of adolescent and young adult CCSs. We therefore aim to evaluate different transition models for Swiss CCSs by assessing their cancer knowledge, cancer worries, self-management skills, and expectations for LTFU care, following transition in two different hospital-based models.

Methods: Within the Aftercare of Childhood Cancer Survivors (ACCS) study, we performed a questionnaire-based survey with a cross-sectional and longitudinal part. We included 5-year CCSs aged >16 years at recruitment who were transitioned to adult care in two hospitals between 2014 and 2021. Here, we report the results of the cross-sectional part. We compared the survivors' cancer knowledge with medical record data and assessed cancer worries (6 questions), self-management skills (15 questions), and expectations (12 questions) by validated scales. We used descriptive statistics, chi-squared test, and t-tests to describe the results.

Results: We analyzed 57 CCSs (response rate 44%), 60% of those were female, had a median age of 9 years at diagnosis and 23 years at the questionnaire. Most CCSs recalled their diagnosis (95%) and exposure to treatment modalities (98%) correctly. CCSs worried the most about potential late effects (47%) and issues with having children in the future (44%). At least 75% of CCSs agreed to 12 of the 15 self-management questions, indicating high self-management skills. The top three expectations included that physicians know the survivors' cancer

10.3389/fonc.2022.946281

history, that visits start on time, and that physicians can always be called in case of questions.

Conclusion: CCSs receiving hospital-based LTFU care have good cancer knowledge and high self-management skills. The identified worries and expectations will help to improve the LTFU care of CCSs who transition to adult care, to further inform and educate survivors and healthcare professionals about and might be relevant for other countries with a similar healthcare system.

KEYWORDS

cancer, child, adolescent, young adult, transition, expectations, worries, self-management skills

Introduction

Advancements in the diagnosis, treatment, and supportive care of children and adolescents diagnosed with cancer contribute to the increasing numbers of long-term childhood cancer survivors (CCSs). In parallel, knowledge on chronic medical conditions, so-called late effects, in long-term CCSs is constantly growing. Data show that the proportion of CCSs suffering from late effects increases as they get older (1–3). As a result, there is a worldwide consensus that most CCSs need long-term and often lifelong follow-up care, as most survivors experience one or more late effects due to the cancer itself or its treatment. Long-term follow-up (LTFU) care aims to reduce the burden of late effects through prevention, early detection, and treatment and to improve CCSs' quality of life. LTFU care is performed risk-adapted and according to national or international recommendations (4–7). When CCSs reach adulthood, LTFU care should be transferred and continued in the adult setting. However, many CCSs discontinue regular follow-up care once they have left the pediatric setting, with increasing drop-off rates with longer time following treatment completion (8–10). This discrepancy between the higher prevalence of late effects in older CCSs and the decrease in adherence to LTFU care is very critical. Therefore, the transition, that is, the movement of LTFU care from the pediatric to the adult setting, is important. The transition is ideally a structured and planned process, coordinated, comprehensive, and multidisciplinary with well-informed healthcare providers. CCSs should also be well informed about their medical history and reasons for LTFU care, enabling them to navigate in the healthcare system on their own (11). The structure of the transition depends on the LTFU care model used. Dixon et al. described five models (12): 1) cancer-center model: care is provided by pediatric oncologists or dedicated survivorship care teams in the cancer center. 2) Shared-care model: care is initially provided by pediatric oncologists or survivorship care teams, which is later handed over to community healthcare providers, with specialty support provided in the cancer center. 3) Disease-specific model: care is tailored to the needs of CCSs at a particular risk, provided in a cancer center (e.g., brain tumor survivors). 4) Risk-stratified model: the individual risk of each CCS to develop late effects defines the place of LTFU care; high-risk CCSs are seen in cancer centers and low-risk CCSs in the community. 5) Consult-based model: care is delivered by community healthcare providers, including specialty support (12). None of these LTFU care models outweighs the others, and the model used depends on the local possibilities. One crucial factor, independent of the model, is education—education of the survivors and healthcare providers (11). Treatment summaries are helpful tools to educate survivors and healthcare providers and an important element of transition. The examples of these treatment summaries are the European "Survivorship Passport" (SurPass) from PanCare and the "Passport for Care" from the Children's Oncology Group (COG) (13, 14). However, being well informed is not the only facilitating factor for a successful transition from a CCS's perspective; further expectations concern communication, organizational aspects, or support for insurance questions (11, 15). Klassen et al. developed and validated scales to assess factors that CCSs perceive as barriers or facilitators during transition (16, 17). Their implementation has been demonstrated to be feasible in the Canadian, Japanese, and Swiss settings (18–20).

Today, we do not know which transition model fits best for Swiss CCSs and what the survivors' cancer knowledge and expectations for LTFU care are. This multicenter, cross-sectional study aims to close this knowledge gap by assessing cancer knowledge, cancer worries, self-management, and expectations for LTFU care in CCSs following transition.

Methods

Study design

This study is part of the Aftercare of Childhood Cancer Survivors (ACCS) study, a prospective, multicenter, observational study, including a cross-sectional and longitudinal part (21). Three pediatric oncology centers were included, each with a different transition model. Clinic A has joint consultations with pediatric and adult oncologists/hematologists being present during the whole consultation for at least two visits. The first visit takes place in the pediatric hospital and the second in the adult hospital. Survivors decide whether further joint consultations happen before LTFU care is handed over to the adult oncologists/hematologists. Clinic B transitions CCSs to the adult clinic during one combined consultation in the pediatric hospital. In both clinics, the pediatric team is available for questions following transition. Additionally, clinic A continuously updates the survivorship care plans following transition. Clinic C transitions CCSs to the family physicians. Eligible CCSs either qualified for the longitudinal (Group 1; transition planned during next annual visit) or the cross-sectional part (Group 2, transition completed at recruitment) of the ACCS study.

Method and data collection

Group 1 survivors received a baseline questionnaire before the annual visit and follow-up questionnaires after 3 and 15 months. Group 2 survivors received one baseline questionnaire at study inclusion (21). Here, we analyzed the cross-sectional part of the ACCS study. This includes the latest questionnaire of each CCS completed following transition, corresponding to the 15-month questionnaire of Group 1 survivors and the baseline questionnaire of Group 2 survivors. The questionnaires were identical, including sections on demographics, cancer knowledge, and validated scales to assess cancer worry, self-management, and expectations for LTFU care (Supplemental Explanation E1) (17). We officially translated the Cancer Worry Scale (CWS) and Self-Management Skill Scale (SMSS) into German and proved their applicability in a previous feasibility study (20). A chart review was performed by pediatric oncologists of each clinic, to collect information on diagnosis, treatment exposure, and the potential risk for late effects. Organ systems at risk were defined according to the COG LTFU guidelines V5.0 (4).

Participants

We recruited 5-year CCSs, who have been diagnosed with cancer according to the International Childhood Cancer Classification third edition (ICCC3) and were aged <18 years at cancer diagnosis and >16 years at recruitment. CCSs further had to be ready for transition (Group 1) or have been transitioned since 2014 (Group 2). We excluded CCSs treated with surgery only, CCSs receiving cancer treatment at time of recruitment or in a palliative situation, and CCSs unable to complete the questionnaire due to cognitive disabilities or language barriers. Recruitment took place between January 2019 and March 2021.

Ethical issues and statistical analysis

Ethics approval was granted by the cantonal ethics committee Ethikkommission Nordwest- und Zentralschweiz (EKNZ), and the study is registered at ClinicalTrials.gov (NCT04284189). We described the study population and the scales mainly descriptively. For the CWS, Klassen et al. provided a scoring system, ranging from 0 to 100, where higher numbers indicate lower degrees of worries (17). For the other scales, we combined the answer options “strongly agree” and “agree” as well as “disagree” and “strongly disagree”. We used the chi-squared test to examine differences in categorical variables and t-test for continuous variables and displayed the results graphically. Except for two questions in the “self-management skill scale”, the proportion of missing data was below 5%. For transparency, the proportion of missing data is displayed for each variable. Data were analyzed using STATA 17.0 (StataCorp. LCC, Version 17. College Station, TX, USA) and p-values <0.05 were considered statistically significant.

Results

Characteristics of participants

Of 130 eligible CCSs from clinic A and B, 64 (49%) participated and 57 (44%) were analyzed. Clinic C did not recruit any CCS during the study period. Seven Group 1 CCSs did not complete the 15-month questionnaire (Figure 1). Two-thirds of CCS (65%) were recruited by clinic A. One fourth (n=17; 28%) were Group 1 CCSs and completed the 15-month questionnaire, all recruited by clinic A. Most participants were female (60%), had a median age of 9 years (IQR 4–14) at diagnosis and 23 years (IQR 21–27) at the questionnaire (Table 1). Survivors from clinic A were younger at diagnosis and older at the questionnaire. The most frequent type of cancer was leukemia (37%), followed by lymphoma (16%) and tumors of the central nervous sys (9%). The distribution of primary cancer types was equal between both clinics.

Current follow-up situation and cancer knowledge

Similar proportions of CCSs were in follow-up care by adult oncologists/hematologists alone (44%) or in joint clinics (39%).

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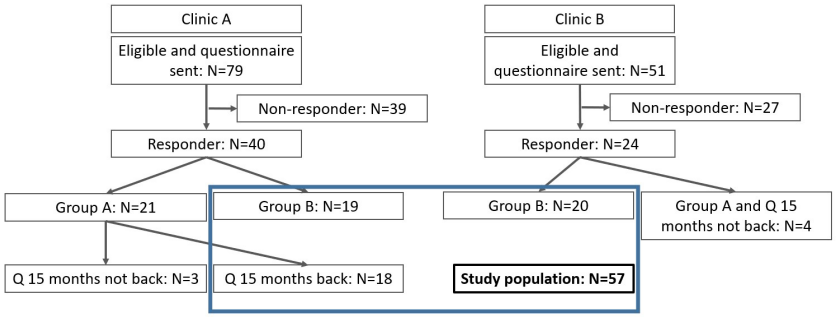

FIGURE 1
Patient Tree, n=57.

TABLE 1 Patient characteristics stratified by recruiting clinic (n=57).

	Clinic A	Clinic B	Total	p-value*
Number of participants, n (%)	37 (65)	20 (35)	57 (100)	
Participation in ACCS				**<0.001**
- Group 2 survivors	20 (35)	20 (35)	40 (70)	
- Group 1 survivors	17 (30)	0	17 (30)	
Sex				0.968
- female	22 (39)	12 (21)	34 (60)	
- male	15 (26)	8 (14)	23 (40)	
Age at diagnosis [years] (reported by physician) Median (IQR)	8 (4–13) range: 1–16	14 (5–16) range: 1–19	9 (4–14) range: 1–19	0.089
Age at survey [years]Median (IQR)	24 (21–27) range 18–35	22 (20–25) range 18–29	23 (21–27) range: 18–35	0.108
Current health status[1]Median (IQR)	9 (8–10) range: 6–10	8 (7–9) range: 5–10	9 (8–9) range: 5–10	0.204
Type of follow-up care				**0.004**
- Joint clinics[2]	21	1	22 (39)	
- Adult hospital alone	11	14	25 (44)	
- Family physician and organs-specific specialists	0	1	1 (2)	
- Family physician only	2	0	2 (3)	
- Follow-up terminated	0	1	1 (2)	
- Other[3]	2	2	4 (7)	
- Missing	1	1	2 (3)	
Type of cancer (reported by physician)				
- Leukemia	15	6	21 (37)	
- Lymphoma	7	2	9 (16)	
- Tumors of the central nervous system	3	1	5 (9)	
- Neuroblastoma	0	1	1 (2)	
- Nephroblastoma	2	2	4 (7)	0.138
- Soft tissue sarcoma	1	3	4 (7)	
- Ewing sarcoma	4	1	5 (9)	
- Osteosarcoma	0	3	3 (5)	
- Germ cell tumors	3	1	3 (5)	
- Other types of cancer	2	0	2 (3)	

*Chi-squared test for categorical variables, t-test for continuous variables; bold values represent significant p-values <0.05.
[1]Subjective assessment of current health status when answering the questionnaire, range from 0 = not at all satisfied to 10 = satisfied a lot.
[2]Joint clinics combine pediatric and adult disciplines for long-term follow-up care.
[3]Orthopedists only, moved abroad, unsure about the current situation.

All CCSs diagnosed with leukemia, lymphoma, nephroblastoma, and osteosarcoma recalled their diagnosis correctly, representing two-thirds of all survivors (n=37, Supplementary Table 1). At least 90% of CCSs stated that they recall their age at diagnosis, age at treatment completion, and the cancer location. Approximately 88% of CCSs felt confident about knowing how often follow-up care visits take place and 67% on their knowledge about potential late effects (Supplemental Table 2A). The agreement between the survivor- and physician-reported treatment exposure was high, with 100% for radiotherapy and bone marrow transplantation and a difference of one survivor each for chemotherapy and surgery (Figure 2A and Supplemental Table 2A). This holds true for the sensitivity analysis, stratified by clinic (Supplemental Tables 2B, C).

The nine questions on potential late effects were answered as "not sure" by 19%–42% of CCS. Approximately 19% of CCSs were unsure about the risk for audiological late effects. In contrast, 42% of CCSs were unsure whether they were at risk for secondary malignancies or not. The organs CCSs considered themselves at risk most frequently included the heart (51%), fertility (39%), endocrine function, and bone health (30% each) (Figure 2B and Supplementary Table 2A). Physicians rated fertility (91%), heart (83%), and secondary malignancies (81%) most frequently. Combining the survivors' answers "not sure" and "yes" resulted in an alignment between the survivors' and physicians' assessments for the heart, visual function, bone health, fertility, memory function, and secondary cancer (Supplemental Figure 1A). Combining the survivors' answers "not sure" and "no" resulted in an alignment between the survivors' and physicians' assessments for lung, hearing, and endocrine function (Supplemental Figure 1B).

Cancer Worry Scale

CCSs fear most about potential late effects (47%) and having children in the future (44%) (Figure 3 and Supplemental Table 3a). One-third of CCSs have cancer always in the back of their minds (35%) or worry about cancer recurrence (28%). The mean CWS score was 62 (SD 19.88; median 60, 95%CI 51–76).

Self-management skill-scale

At least 90% of CCSs agreed to 6 of the 15 SMSS statements (Q1–3, Q8, Q10, Q14; Table 2). Between 75% and 90% of CCSs agreed to further six statements (Q4, Q6, Q7, Q12, Q13, Q15). Two questions with less than 75% agreement indicate that the parents' presence is preferred during follow-up visits (Q9, Q11).

Expectation scale

Five statements were considered important by at least 75% of survivors, dealing with prepared and engaged physicians (S1, S6) and administrative issues (S2, S3, S7). Least important were statements on personal relationships with treating physicians, including the statement that visits do not always have to be with the same physician (Table 3). The answers to the questions Q8, Q10, and Q11 indicated that CCSs seem to have the feeling that the adult setting is more distant and less personal than in pediatric oncology.

Discussion

Our study shows that CCSs enrolled in hospital-based transition models were well informed about their diagnosis and treatment, which were validated by physicians' information, with less congruence between survivors' and physicians' perception on organs at risk for late effects.

The survivors' cancer knowledge is as high as in Canadian and American survivors. In a Canadian study, 93.6% of 250 survivors aged 15–26 years from three clinics recalled their diagnosis correctly (22). In an American case–control study, 98% of 87 survivors from a survivorship clinic or their parents recalled their diagnosis correctly and 90% of survivors or their parents in routine follow-up care (controls) (23). CCSs from our cohort were older at the questionnaire than the Canadian survivors (median 23 years versus median 17 years) and had a longer follow-up than the American survivors (median 13.5 years versus mean 5.2 years). CCSs from all three cohorts showed a high knowledge on treatment received, which might be explained by the structured LTFU care and the involvement of specialized healthcare professionals: transfer to specific LTFU care between 6 and 24 months from treatment completion in the Canadian cohort; evaluation in a survivorship clinic in addition to routine care in the US cohort; and transition to hospital-based joint consultations or adult oncology alone at around 18 years of age in our cohort. This highlights that different hospital-based approaches are equally good in imparting knowledge.

Swiss survivors showed moderate cancer worries, with a higher CWS score than two Canadian cohorts [mean CWS score 62 (SD 19.88) versus 50.6 (SD 18.4) and 57.8 (SD 19.4)] (18, 24). A Japanese study used the same CWS without reporting the score (19). The proportion of survivors that either agree or strongly agree to each of the six statements varies largely between the cohorts with Swiss survivors having the least cancer worries (Supplemental Table 3B). As our results are comparable with those from the preceding feasibility study, the lower worries are reproducible (18). Low cancer worries might be the result of well-informed survivors, including knowledge on where to go in case of symptoms or uncertainties, or not well-

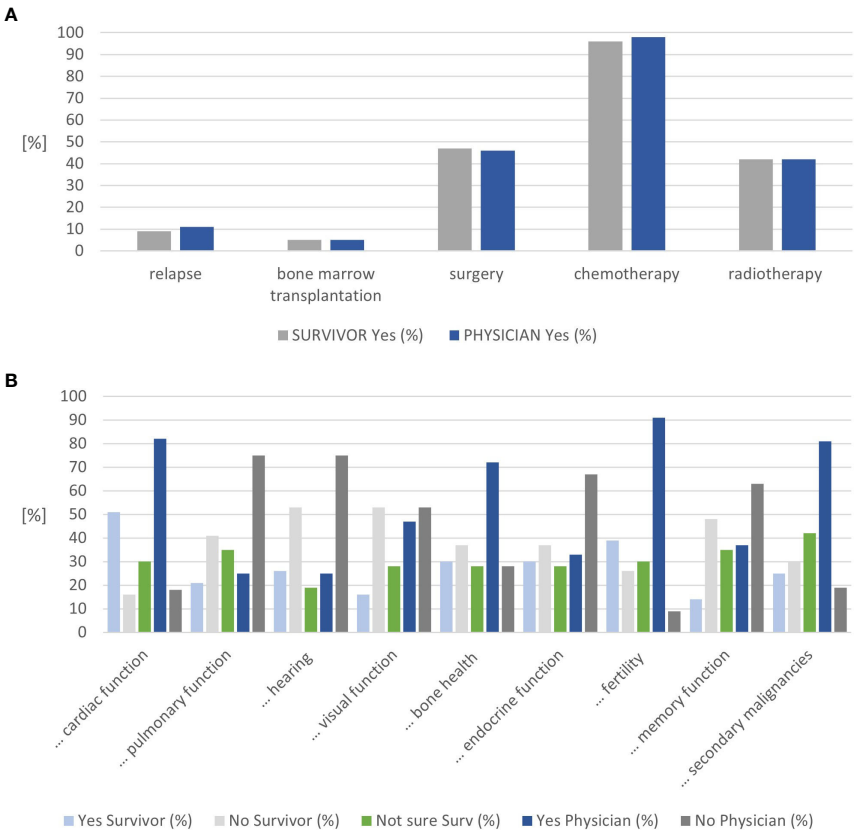

FIGURE 2
Results on cancer knowledge. **(A)** Congruence on treatment exposure mentioned by childhood cancer survivors and physicians. **(B)** Congruence on organ systems considered at risk by childhood cancer survivors and physicians (n=57).

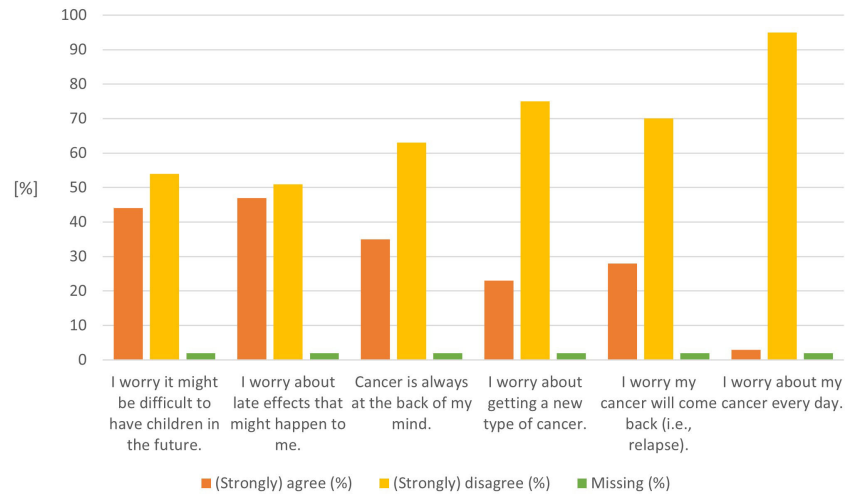

FIGURE 3
Results from the validated Cancer Worry Scale (n=57).

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TABLE 2 Results from the Self-Management Skills Scale, shaded areas indicating at least 75% agreement with the respective statement (n=57).

	Strongly agree n (%)	Agree n (%)	Disagree n (%)	Strongly disagree n (%)	Missing n (%)
Q1 I answer a doctor or nurse's questions.	39 (68)	15 (26)	0	1 (2)	2 (4)
Q2 I participate in making decisions about my health.	31 (54)	22 (39)	1 (2)	0	3 (5)
Q3 I make sure I go to all my doctor's appointments.	45 (79)	8 (14)	1 (2)	0	3 (5)
Q4 I ask the doctor or nurse questions.	17 (30)	33 (58)	5 (9)	0	2 (3)
Q5 I talk to a doctor or nurse when I have health concerns.	16 (28)	26 (46)	11 (19)	2 (3)	2 (3)
Q6 I talk about my medical conditions to people when I need to	18 (32)	27 (48)	8 (14)	2 (3)	2 (3)
Q7 I am in charge of taking any medicine that I need	37 (65)	11 (19)	3 (5)	0	6 (11)
Q8 I know how to contact a doctor if I need to.	34 (60)	19 (34)	2 (3)	0	2 (3)
Q9 I prefer it when a doctor speaks to me instead of my parent(s).	18 (32)	20 (35)	12 (21)	4 (7)	3 (5)
Q10 I can briefly describe my medical history when asked	29 (51)	22 (39)	3 (5)	0	3 (5)
Q11 I prefer to see a doctor or nurse without any parent(s) with me	21 (37)	18 (32)	11 (19)	4 (7)	3 (5)
Q12 I know how to access medical care when I travel.	21 (37)	27 (47)	6 (11)	0	3 (5)
Q13 I book my own doctor's appointments	36 (63)	12 (21)	7 (13)	0	2 (3)
Q14 I know the type of medical insurance I have.	46 (81)	9 (16)	0	0	2 (3)
Q15 I fill my own prescriptions when I need medicine	32 (57)	16 (28)	3 (5)	2 (3)	4 (7)

informed survivors, not concerned due to the lack of knowledge. As 94% of CCSs from our cohort know how to contact a doctor if they need to, we conclude that they are well informed, especially on how to navigate in the Swiss healthcare system. Including 5-year survivors only might explain the relatively low proportion of survivors worrying about cancer recurrence, as the risk of relapse is rather low in this population, again indicating well-informed survivors.

Overall, Canadian survivors showed higher self-management skills than Swiss CCSs, as a higher proportion of Canadian CCSs agreed to 11 of the 15 questions (Supplemental Table 4) (18). However, survivors from our cohort show higher self-management skills in administrative fields (Q13–Q15), including booking doctor's appointments, knowing the medical insurance, or filling their own prescriptions. This might be explained by the Swiss insurance system, as adolescents must take care of their own health insurance at the age of 18. The lowest agreement between the Swiss and Canadian cohort concerns the parental involvement (Q9, Q11), where Swiss survivors favor the parental involvement. These results show that childhood cancer survivors have to learn to become independent from their parents when they grow older. They are often used to the fact that the parents take care of the appointments or answer the physicians' questions. These answers also highlight that certain areas of long-term follow-up care have to improve to empower the survivors better.

TABLE 3 Results from the Expectation Scale, shaded areas indicating at least 75% agreement with the respective statement (n=57).

	Expectation				
When leaving the children's hospital I expect that…	**Strongly agree n (%)**	**Agree n (%)**	**Disagree n (%)**	**Strongly disagree n (%)**	**Missing n (%)**
S1… my after care physician knows my cancer history.	42 (74)	13 (23)	0	0	2 (3)
S2… the visit starts on time	17 (30)	37 (65)	1 (2)	0	2 (3)
S3… I get a call when I miss an appointment	16 (28)	27 (48)	7 (12)	4 (7)	3 (5)
S4… I am always seen by the same physician	15 (27)	24 (42)	12 (21)	4 (7)	2 (3)
S5… I get a reminder before each visit	13 (23)	16 (28)	17 (30)	8 (14)	3 (5)
S6… I can always call my physician in case of questions	17 (30)	35 (62)	3 (5)	0	2 (3)
S7… other examinations for follow-up care take place on the same day	22 (39)	21 (37)	11 (19)	1 (2)	2 (3)
S8… my parents can come to the visit	6 (11)	19 (33)	15 (26)	14 (25)	3 (5)
S9… thy physician takes care of all my medical problems	14 (25)	19 (33)	17 (30)	4 (7)	3 (5)
S10 … my follow-up care physician becomes like a friend	3 (5)	11 (20)	22 (39)	19 (33)	2 (3)
S11… my follow-up care physician team spends a lot of time with me.	1 (2)	6 (11)	35 (61)	13 (23)	2 (3)
S12… I like going to my follow-up appointments	6 (11)	21 (37)	21 (37)	6 (10)	3 (5)

Most factors Swiss survivors expect from their follow-up appointments are related to physicians' knowledge about their history and structural aspects of the clinical visits. Knowing a survivors' history is a key factor and gives confidence in the relationship between survivors and physicians (11). Having separate LTFU care consultations with dedicated physicians, experienced with possible late effects and the issues of survivors, may be beneficial. Further, LTFU care clinics often have the possibility to organize all examinations on 1 day. Starting the visit on time might also be better feasible in LTFU care clinics, separated from children undergoing active treatment. In summary, it seems feasible to implement the items considered important by the CCSs in clinic routines without much effort.

Our findings have some limitations and strengths. Absent recruitment from clinic C resulted in the analysis of two hospital-based models only, and no conclusions are possible about other transition models, especially the transition to family physicians. Clinic C was not able to identify the survivors transitioned to the family physicians between 2014 and 2021, and obviously no survivor was eligible for transition during the study period. Further, clinic B recruited Group 2 survivors only, resulting in more survivors with a longer follow-up period. Through participation bias, the results might not be representative for all Swiss CCS, either because only those with better knowledge or less late effects participated or only those with less knowledge or more late effects. As the survivors' characteristics are comparable to other CCS cohorts, we consider the results representative for long-term CCS. The sample size made the analysis of knowledge and needs of subgroups impossible, such as separate tumor entities or treatment exposures. The participation rate was lower compared to large CCS cohorts (25–27). However, considering that some CCSs left pediatric care many years ago, the response rate of 44% is still high and comparable to other studies with the same approach (18, 19). The long follow-up period of median 14 years (IQR 11–19) is a further strength.

Conclusion

Hospital-based follow-up care models result in high cancer knowledge and moderate cancer worries and self-management skills. Changeable structural conditions could be identified. An extension of the ACCS study is planned to evaluate the transition to family physicians.

Data availability statement

Data can be made available upon request to the authors. Requests to access the datasets should be directed to maria.otth@ksa.ch.

Ethics statement

This study involving human participants was reviewed and approved by the Cantonal Ethics Committee (EKNZ). Written informed consent to participate in this study was provided by the participants.

Author contributions

Conceptualization: KS and MO; Data collection: MO, SD, and TD-F; Formal analysis: MO; Funding acquisition: KS; Writing—original draft: MO; Writing—review and editing: KS, SD, TD-F, and MO. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the Swiss Cancer Research, grant number HSR-4359-11-2017.

Acknowledgments

We thank all childhood cancer survivors for participating in the ACCS Project.

Conflict of interest

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

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.946281/full#supplementary-material

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frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 06 September 2022
DOI 10.3389/fonc.2022.906194

Check for updates

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 28 March 2022
ACCEPTED 02 August 2022
PUBLISHED 06 September 2022

CITATION
Sorteberg AL, Halipi V, Wickström M and Shirazi Fard S (2022) The cyclin dependent kinase inhibitor p21$^{Cip1/Waf1}$ is a therapeutic target in high-risk neuroblastoma. *Front. Oncol.* 12:906194. doi: 10.3389/fonc.2022.906194

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The cyclin dependent kinase inhibitor p21$^{Cip1/Waf1}$ is a therapeutic target in high-risk neuroblastoma

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Platinum-based chemotherapies such as cisplatin are used as first-line treatment for the paediatric tumour neuroblastoma. Although the majority of neuroblastoma tumours respond to therapy, there is a high fraction of high-risk neuroblastoma patients that eventually relapse with increased resistance. Here, we show that one key determinant of cisplatin sensitivity is phosphorylation of the cyclin-dependent kinase inhibitor p21$^{Cip1/Waf1}$. A panel of eight neuroblastoma cell lines and a *TH-MYCN* mouse model were investigated for the expression of p21$^{Cip1/Waf1}$ using RT-qPCR, Western blot, and immunofluorescence. This was followed by investigation of sensitivity towards cisplatin and the p21$^{Cip1/Waf1}$ inhibitor UC2288. Whereas the cell lines and the mouse model showed low levels of un-phosphorylated p21$^{Cip1/Waf1}$, the phosphorylated p21$^{Cip1/Waf1}$ (Thr145) was highly expressed, which in the cell lines correlated to cisplatin resistance. Furthermore, the neuroblastoma cell lines showed high sensitivity to UC2288, and combination treatment with cisplatin resulted in considerably decreased cell viability and delay in regrowth in the two most resistant cell lines, SK-N-DZ and BE(2)-C. Thus, targeting p21$^{Cip1/Waf1}$ can offer new treatment strategies and subsequently lead to the design of more efficient combination treatments for high-risk neuroblastoma.

KEYWORDS

p21(Thr145), UC2288, small molecular inhibitor, chemo-resistance, combination treatment, oncogene

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumour in early childhood. It is a heterogenous neoplasia with clinical presentation ranging from spontaneous regression of metastatic disease to a rapidly progressive course (1, 2).

High-risk NB patients, accounting for 50% of all children diagnosed with NB, often present with unresectable primary lesions and/or multiple metastases (1). More importantly, despite intensive multimodal treatment, relapse rates are as high as 50% and are frequently characterised by therapy resistance and intra-tumour diversity, making high-risk NB difficult to cure (3, 4).

In order to combat development of resistant cells, novel targeted strategies are being implemented for high-risk NB, including small-molecule inhibitors of anaplastic lymphoma kinase (ALK), radiolabelled somatostatin analogues, and monoclonal antibodies against the antigen GD2 (5, 6). Even though the overall survival of high-risk NB patients has increased following anti-GD2 immunotherapy, one third of the patients experience treatment failure within the first two years of the treatment, mostly due to a variety of immune evasion strategies (7).

Despite challenges, chemotherapy (CT) continues to be the cornerstone of systemic NB treatment. The platinum complex cisplatin is commonly included in first line therapies as adjuvant therapy, with the aim of inducing cytotoxicity and apoptosis (1, 8, 9). Unfortunately, the effect is not exclusive to cancer cells, and cisplatin-induced side effects include hearing loss, neuro- and/or renal-toxicity or bone marrow-suppression (1, 9, 10). A major role in the response to cisplatin has been ascribed to the tumour cell´s sensitivity to the drug. Recently, the expression of the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ (p21) has been linked to cisplatin resistance in testicular cancer (11) and in ovarian cancer (12).

The p21 protein is transcriptionally regulated by p53-dependent and –independent pathways and is a key regulator of cell fate. Based on the intracellular localization, the p21 protein shows pleiotropic effects on cell growth and apoptosis in both malignant and non-malignant cells and tissue (13–16). In the nucleus, the p21 protein can function as a tumor suppressor by acting as a cdk2 inhibitor thereby initiating cell cycle arrest in response to DNA damage (17), and also as an oncogene by increasing the assembly of CDK4/6 and cyclin D that initiates entry into the S-phase (18, 19). The cytoplasmic localization of p21 is mainly driven by Akt mediated phosphorylation on Thr145 (p-p21) (20) and when located in the cytoplasm, p-p21 prevents apoptosis (21, 22), further establishing its role as an oncogene.

Few small molecule inhibitors of p21, with the aim to inhibit p21 for potential cancer therapy, have been reported so far. These include butyrolactone I (23), LLW10 (24), sorafenib (25), and the recently developed small molecular inhibitor UC2288, which was developed through modification of sorafenib (26). UC2288, which functions independently of p53, is a more specific p21 inhibitor than sorafenib and attenuates the p21 protein at the level of transcription or post-transcription. In addition, UC2288 markedly decreases cytosolic p21 protein levels, thereby inhibiting the ability of p21 to convey an anti-apoptotic function (26).

The possible role of p21 as an oncogene has previously not been studied in high-risk NB. Therefore, we have here mapped the endogenous expression of p21, both un-phosphorylated and phosphorylated, in a panel of eight high-risk NB cell lines and one NB mouse model. The effect of UC2288, either alone or in combination with cisplatin, was examined to investigate how functional modulation of p21 and p-p21 may alter NB cell viability. Here we report that increased cisplatin resistance was correlated to p-p21, and that combination treatment with UC2288 and cisplatin considerably decreased tumour cell viability. These results suggest an important role for p21 and p-p21 in preventing cisplatin-induced cell death in high-risk NB.

Material and methods

Cell Culturing

BE(2)-C and Kelly were obtained from ATCC (Manassas, VA, USA). SK-N-SH, SH-SY5Y, IMR-32, SK-N-AS, SK-N-FI, SK-N-DZ, and MRC5 were a kind gift from professor Per Kogner at Karolinska Institute. HL-60 was a kind gift from Dr. Mohammad Hojjat Farsangi at Karolinska Institute. Eight high-risk NB cell lines were used, where three were *TP53* wild type (wt) and five were *TP53* mutated (mut) in order to compare between *TP53* dependent and independent expression of p21. The non-tumourigenic fibroblast cell line MRC5 and the acute promyelocytic leukemia cell line HL-60 were included as controls (Supplementary Table S1). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin (all from Life Technologies Inc, Thermo Fisher Scientific, Stockholm, Sweden). Cells were incubated at 37°C with 5% CO2 and high humidity.

Mouse model

The transgenic *TH-MYCN* animals were obtained from the Mouse Model of Human Cancer Consortium Repository and kept on a 129X1/SvJ background (27). Tumours from the transgenic neuroblastoma *TH-MYCN* model are morphologically and phenotypically similar to human high-risk NB. All mice were housed in standard cages in a temperature- and humidity-controlled room with 12-hour light/12-hour dark cycles and ad libitum access to water and food. Genotyping was performed from ear tissue biopsies using qPCR with specific probes designed for wild-type and the

MYCN transgene (Transnetyx, Cordova, TN, USA). We here used tumours from hemizygous mice ($MYCN^{+/-}$) for immunofluorescent staining for p21 and p-p21 according to the immunofluorescent protocol description.

Western blot analysis

Twenty-four hours after plating the protein from the NB cell lines, the MRC5, and the HL-60 cell lines were extracted for the baseline data. The protein extraction was done by using RIPA buffer (Thermo Fisher Scientific, Stockholm, Sweden) supplemented with a protease and phosphatase inhibitor (Thermo Fisher Scientific, Stockholm, Sweden) according to the manufacturer's recommendations, separated electrophoretically and blotted on a nitrocellulose membrane (Bio-Rad, California, USA) using standard procedures. All samples were loaded at 20 µg for equal loading. The p21 protein was detected using an anti-p21 antibody (Cell Signaling, #2947, 1:500) or an anti-p-p21 (T145) antibody (Abcam, #ab47300, 1:250), and Cleaved-Caspase3 (C-Casp3) was detected using an anti-C-Casp3 antibody (Cell Signaling, #9661, 1:1000). GAPDH was stained with anti-GAPDH antibody (Thermo Fisher, #39-8600, 1:4000). Protein bands were visualized with secondary antibodies diluted 1:15,000 (IRDye680 and IRDye800, LI-COR, Nebraska USA) using a ChemiDoc MP imaging system (Bio-Rad, California, USA) or a LI-COR imaging system (LI-COR Biosciences UK Ltd). Analysis of band intensity was performed using ImageJ. All experiments were done in triplicate or quadruplicate, except for the HL-60 experiments which were done in duplicates.

Real time quantitative polymerase chain reaction

Each cell line in logarithmic growth was plated onto petri dishes and incubated for 24 hours before samples for RT-qPCR were harvested. mRNA was harvested according to the RNeasy Mini Kit protocol (Qiagen, Hilden, Germany). ß-mercaptoethanol was added at a volume of 10 µl/ml of Buffer RLT. mRNA concentration was measured using Nanodrop™ (Thermo Fisher Scientific, Stockholm, Sweden). Reverse transcription of mRNA into cDNA was performed according to the Quantitect® Reverse Transcription manufacturer protocol (Qiagen, Hilden, Germany). RT-qPCR was performed following QuantiText® SYBR® Green PCR manufacturer protocol (Qiagen, Hilden, Germany). The primer sequences used can be found in Table 1. HPRTI was used as a housekeeping gene for the NB cell lines as this has been found to be one of the most stably expressed genes in NB (28), while GAPDH was used as a housekeeping gene for the MRC5 and the HL-60 cell lines. HL-60 was used as the calibrator sample to calculate relative expression. All experiments were done in quadruplicates.

Immunofluorescence

Each cell line in logarithmic growth was plated onto round 13 mm #1 coverslips (VWR, Stockholm, Sweden) in petri dishes and incubated for 24 hours before being fixated for 15 minutes using 4% paraformaldehyde (Merck, Molsheim, France). TNB buffer (0.5 g blocking reagent [PerkinElmer, Stockholm, Sweden] to 100 mL TBS buffer [Tris/NaCl pH 7.4]) block was added for 30 min at room temperature. Primary antibody diluted in 0.3% Triton X-100, 0.1% NaN_3 in 1xPBS was added and the cells were incubated overnight at +4°C. The primary antibody was then removed, and cells were washed 3x5 min with 1xPBS before the secondary antibody, diluted in TNB buffer, was added. The cells were kept at room temperature for 2 hours before the secondary antibody was removed and the cover slips were washed 3x5 min with 1xPBS. The cells were mounted with Prolong Gold antifade with DAPI (Thermo Fisher Scientific, Stockholm, Sweden) to stain the cell nuclei. A Metafer® Slide Scanning Platform (version 3.13.4, Metasystems, Heidelberg, Germany) was used to analyse the cells. The spatial localization of either the p21 or p-p21 expression was done by optical inspection by two independent observers. The mean intensity (mean fluorescence in arbitrary units per cell ± SD) was provided by the Metafersystem software and the low, intermediate, or high expression levels were based on equal range distributions of the values. Following the protocol for immunofluorescence, a triple staining was performed on all eight cell lines using antibodies for p21, the DNA damage marker phosphorylated ataxia-telangiectasia mutated (pATM), and the replication marker Ki67. All experiments were done in triplicate. Antibodies used can be found in Table 2.

TABLE 1 Primer sequences for HPRTI (housekeeping gene for NB cell lines), GAPDH (housekeeping gene for MRC5 and HL-60) and p21, respectively.

Target gene	Primer sequence forward	Primer sequence reverse
HPRTI	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
p21	AGGTGGACCTGGAGACTCTCAG	TCCTCTTGGAGAAGATCAGCCG

The primer annealing temperature used was 60°C.

TABLE 2 Antibodies used for immunofluorescence.

Antibody	Code	Company	Dilution
Anti-p21$^{Waf1/Cip1}$ (12D1)(rabbit)	2947	Cell Signaling Technologies	1:1000
Anti-p21$^{Waf1/Cip1}$ (phospho T145) (rabbit)	ab47300	Abcam	1:1000
Anti-ATM (phospho S1981) (mouse)	ab36810	Abcam	1:500
Anti-Ki67 (chicken)	ab254123	Abcam	1:1000
Anti-C-Casp3 (Asp175) (rabbit)	9661	Cell Signaling Technologies	1:1000
Alexa flour 594 Anti-Rabbit IgG	711-585-152	Jackson ImmunoResearch	1:800
Cy2 Anti-Mouse IgG	115-225-146	Jackson ImmunoResearch	1:400
Alexa Fluor 647 Anti-Chicken IgY (IgG)	703-605-155	Jackson ImmunoResearch	1:400

Inhibition study

Twenty-four hours after plating of Kelly and SH-SY5Y, UC2288 at a dose of 10 μM (Merck, Molsheim, France) diluted in dimethyl sulfoxide (DMSO) was added for 2 hours, 4 hours or 6 hours before the cells were fixated for 15 min with 4% paraformaldehyde and treated according to the protocol for immunofluorescence, or collected for protein or mRNA according to the protocol for either Western blot or RT-qPCR. UC2288 was added at a concentration of 10 μM based on the literature (29). All experiments were done in triplicate or quadruplicates. The BE(2)-C cell line was treatment for 24 hours with mock (DMSO), cisplatin at a dose of IC_{50} (Accord Healthcare Limited, Middlesex, UK), UC2288 at a dose of 10 μM or a combination of cisplatin and UC2288 followed by fixation for 15 min with 4% paraformaldehyde and treated according to the protocol for immunofluorescence or collected for protein according to the protocol for Western blot. All experiments were done in quadruplicates.

Cell viability assay

Dose–response curves for cell viability were obtained after treatment for 72 hours with either UC2288 or cisplatin, in eight NB cell lines and one fibroblast cell line, MRC5, which was used as a non-tumourigenic control for drug toxicity. UC2288 was added as a single agent at concentrations of 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM, 0.1 μM, 0.05 μM or 0.01 μM and cisplatin at concentrations of 100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM, 0.1 μM or 0.05 μM. For the combination treatments, cisplatin was added at concentrations of 100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM, 0.1 μM or 0.05 μM either alone or in combination with UC2288 at a concentration of either 10 μM or 1 μM. The plates were incubated at 37°C, 5% CO_2. Seventy-two hours after treatment, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed where 20 μl CellTiter 96® Aqueous One Solution Assay (Promega, Stockholm, Sweden) was added to each well. Plates were incubated for 2.5 hours and metabolic activity was analysed in the microplate reader FLUOstar Omega (BMG LABTECH, Ortenberg, Germany) by measuring absorbance at 490 nm and 690 nm. All treatments were done in triplicate.

Confluency assay

SK-N-FI (20×10^3 cells), Kelly (10×10^3 cells), SK-N-DZ (10×10^3 cells) and BE(2)-C (5×10^3 cells) were seeded in 200 μl medium/well in a 96 well plate, and the edges were filled with medium to avoid edge effects. Twenty-four hours after seeding each cell line was treated with the corresponding IC_{50} value for cisplatin and 10 μM UC2288 for 48 hours. DMSO (mock) was used as negative controls. The plates were scanned by an IncuCyte S3 Live® Cell Analysis System (Essen Bioscience, Welwyn Garden City, UK) 48 hours post treatment followed by medium change. The plates were thereafter scanned followed by medium change every 96 hours for a total of 30 days. The proliferation was determined by measuring the cell confluency. Once the cell lines reached 100% confluency, they were maintained for the duration of the experiment. Each treatment was done in 5-10 replicates and the data are presented as mean ± Standard Error of the Mean (SEM).

Combination index analysis

To estimate the effects of the combination treatments the median-effect method of Chou (Chou-Talalay method) (30) was applied to compute combination index (CI) with the ComboSyn software (http://www.combosyn.com; ComboSyn, Inc). In general, a CI of <1 is considered a positive and a CI of >1 a negative combined effect. More specifically, CI<0.70 was defined as synergy, CI>1.45 as antagonism, and values in between as additive effects, according to the recommendations of the ComboSyn software.

Statistical analysis

Following the MTS-assay, the data was analysed to obtain the IC_{50} value for each of the single treatments or for the combination treatment. The data was logarithmically transformed and normalized against 0% (no cell viability) and 100% (maximum cell viability) controls for the combination treatments. For determining IC_{50} values for the single treatments, the nonlinear regression analysis method log (inhibitor) vs normalized response was used. For determining IC_{50} values for combination treatment, the nonlinear regression analysis log(inhibitor) vs normalized response – variable slope was used. Linear regression analysis with Pearson's correlation coefficient was used to investigate a possible relationship between the IC_{50} values of UC2288 and cisplatin, and between the fraction of p21 positive cells with either UC2288 or cisplatin. All analyses were performed in Graphpad Prism (version 9.3.1). Western blot data are presented as mean ± standard deviation (SD). Immunofluorescence data were analysed in RStudio and the percentage of positive cells followed by the mean ± standard deviation (SD) were calculated for each combination. Data was analysed using Student's t-test or one-way ANOVA with Tukey *post hoc* test, as indicated in the text. Graphpad Prism (version 9.1.0) was used for generation of graphs.

Results

Investigation of endogenous p21 and p-p21 expression

Real time-qPCR was used in order to determine the baseline (i.e. without prior treatment) mRNA expression level of p21 (CDKN1A) within eight high-risk NB cell lines and one non-tumourigenic fibroblast cell line, MRC5. The MRC5 cell line had the highest p21 mRNA expression compared to the remaining cell lines ($p<0.0001$) (Figure 1A). Comparing the expression of p21 mRNA within *TP53* wt cell lines or *TP53* mut cell lines showed that among the *TP53* wt cell lines SK-N-SH had the highest p21 mRNA expression followed by IMR-32 and among the *TP53* mut cell lines SK-N-FI and Kelly showed the highest p21 mRNA expression (Figure 1A). These results were further investigated using Western blot for analysis of protein expression. MRC5 showed the highest expression of p21 among the tested cell lines ($p<0.001$) (Figures 1B). The NB cell lines had low expression of p21, with the expression being below detection levels in two cell lines, BE(2)-C and SK-N-DZ (Figures 1B, C). Investigation of p-p21 showed that the expression was highest in MRC5 among the tested cell lines ($p<0.001$) (Figures 1B, C). The NB cell lines had low expression of p-p21, with the expression being below detection levels in four cell lines, IMR-32, SH-SY5Y, Kelly, and SK-N-DZ (Figures 1B, C).

In order to investigate the possible occurrence of p21 and p-p21 expressing cells that might not be detected in bulk analysis we used the Metafer® slide scanning system, which facilitates high-throughput quantitative immunofluorescence microscopy. This allowed the determination of p21 and p-p21 protein expression in single cells. In line with the Western blot data, the fibroblast cell line, MRC5 showed the highest fraction of p21 positive cells (45.3% ± 2.7, $p<0.001$) among all the tested cell lines (Figure 1D). However, unlike the Western blot data, p21 expressing cells were detected in all the NB cell lines. This is in line with the RT-qPCR data (Figure 1A). Moreover, comparing the expression of p21 within *TP53* wt cell lines or *TP53* mut cell lines showed that the fraction of p21 positive cells was higher in the *TP53* wt cell lines SK-N-SH (13.1% ± 2.2, $p<0.05$) and IMR-32 (14.2% ± 6.0, $p<0.01$), compared to SH-SY5Y (4.7% ± 0.9). In the *TP53* mut cell lines, the fraction of p21 positive cells was generally lower with SK-N-FI (10.2% ± 0.9, $p<0.01$) having a significantly higher fraction of positive cells compare to BE(2)-C (1.5% ± 0.3) and SK-N-DZ (0.1% ± 0.1). Cells positive for p21 were also detected in SK-N-AS (5.7% ± 0.6) and Kelly (4.9% ± 0.5) (Figure 1D). Furthermore, the p21 protein was predominantly localized in the nuclear compartment, with no detectable staining of cytoplasmic p21, in all the tested cell lines (Supplementary Figure S1A).

Investigation of the p-p21 expression showed that the fibroblast cell line MRC5 had a low fraction of p-p21 positive cells (14.0% ± 12.3, $p<0.05$) compared to the three cell lines with the highest expression, SH-SY5Y (78.6% ± 17), SK-N-FI (93.9% ± 9.1), and SK-N-DZ (97.6% ± 2.2) (Figure 1E). Moreover, SH-SY5Y (78.6% ± 17, $p<0.01$) had the highest fraction of positive cells among the *TP53* wt cell lines SK-N-SH (8.6% ± 8.8) and IMR-32 (2.1% ± 2.2). Among the *TP53* mut cell lines, SK-N-FI (93.9% ± 9.1) and SK-N-DZ (97.6% ± 2.2) had significantly higher fractions of p-p21 positive cells compared to SK-N-AS, Kelly, and BE(2)-C cell lines which had moderate expression (29.9% ± 46.6, 14.2% ± 15.9, and 20.6% ± 19.0, respectively) (Figure 1E).

Unlike the p21 expression p-p21 was observed in both the cytoplasm and in the nucleus. Quantification of p-p21 expression showed that the p-p21 protein was predominantly localized in the cytoplasm for MRC5 and BE(2)-C, which displayed high expression. Moderate cytoplasmic expression was observed in SK-N-SH, SK-N-FI, and SK-N-AS, followed by low or no detectable cytoplasmic staining in IMR-32, SH-SY5Y, Kelly, and SK-N-DZ (Figure 1F; Supplementary Figure S1B). The three cell lines displaying the highest fraction of p-p21 positive cells, SH-SY5Y, SK-N-FI, and SK-N-DZ, all had predominant nuclear expression of the protein (Figure 1F). However, these cells did not show high expression of p-p21 when investigated with Western blot, instead the results from the Western blot correlated to the cytoplasmic expression of p-p21 where cells with high or moderate expression showed a band (Figure 1B, F). There seems therefore to be a discrepancy between the p-p21 antibody when used either with Western blot or with immunofluorescence where Western blot mainly

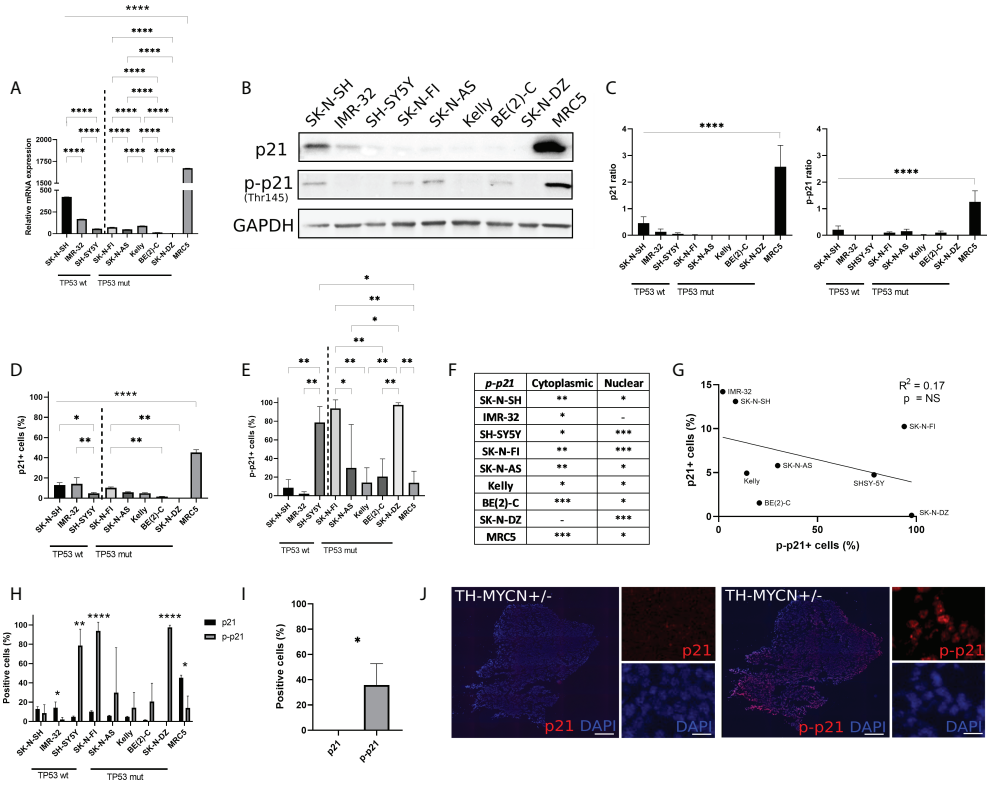


FIGURE 1
Endogenous p21 and p-p21 expression. **(A)** Relative expression of p21 mRNA (CDKN1A), normalized to HL-60. The MRC5 cell line showed the highest endogenous expression compared to the remaining cell lines. Among the NB cell lines the *TP53* wt cell line SK-N-SH showed the highest endogenous expression compared to the remaining cell lines. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, not significant p>0.05. One-way ANOVA with Tukey *post hoc* test. Mean ± SEM, n=4. **(B)** Western blot analysis showing protein expression of p21 (21 kDa), p-p21 (Thr145) (32 kDa), and loading control GAPDH (37 kDa) in NB cell lines and MRC5. **(C)** Western blot analysis showed the highest endogenous expression of p21 or p-p21 in MRC5 compared to the remaining cell lines. **** = p<0.0001, not significant p>0.05. One-way ANOVA with Tukey *post hoc* test. Mean ± SD, n=3-4. **(D)** Fraction of p21 expressing cells in each cell line. The highest fraction of p21 positive cells was observed in the MRC5 cell lines. Among the NB cell lines, the *TP53* wt cell lines showed generally a higher fraction of cells positive for p21 compared to the *TP53* mut cell lines. * = p<0.05, ** = p<0.01, **** = p<0.0001, not significant p>0.05. One-way ANOVA with Tukey *post hoc* test. Mean ± SD, n=3. **(E)** Fraction of p-p21 (Thr145) expressing cells in each cell line. Three cell lines, SH-SY5Y, SK-N-FI, and SK-N-DZ showed the highest fraction of p-p21 positive cells. * = p<0.05, ** = p<0.01, not significant p>0.05. One-way ANOVA with Tukey *post hoc* test. Mean ± SD, n=3. **(F)** Investigation of spatial localization of the p-p21 protein. The BE(2)-C and MRC5 cell lines showed highest cytoplasmic p-p21 expression, whereas SH-SY5Y, SK-N-FI, and SK-N-DZ showed the highest nuclear p-p21 expression. - = no expression, * = low expression, ** = moderate expression, *** = high expression. n=3. **(G)** Fraction p21 versus p-p21 positive cells and the correlation between the two. A trend in the correlation was observed, however it was not significant. Linear regression with Pearson's correlation coefficient, NS= not significant p>0.05. n=3. **(H)** Comparison between the fraction of p21 and p-p21 expressing cells in each cell line. IMR-32 and MRC5 had a lower fraction of p-p21 positive cells compared to p21 positive cells, whereas SH-SY5Y, SK-N-FI, and SK-N-DZ had a higher fraction of p-p21 positive cells compared to p21 positive cells. * = p<0.05, ** = p<0.01, **** = p<0.0001, not significant p>0.05. Student´s t-test. Mean ± SD, n=3. **(I)** The *TH-MYCN* heterozygote mouse model did not show p21 positive cells, whereas 35.9% ± 16.8 of tumour cells where positive for p-p21. * = p<0.05. Student´s t-test. n=3. **(J)** representative pictures of p21 and p-p21 staining on tumour tissue in the *TH-MYCN* heterozygote mouse model. Scale bar 200 µm and 20 µm, respectively.

detects cytoplasmic expression and immunofluorescence detects both cytoplasmic and nuclear expression. In order to investigate the specificity of the p21 and the p-p21 antibodies the HL-60 cell line was used since this cell line has the lowest expression of p21 mRNA among a panel of 69 tested cell lines (https://www.proteinatlas.org/ENSG00000124762-CDKN1A/cell+line). No p21 or p-p21 staining was detected in the HL-60 cell line when investigated with Western blot and immunofluorescence validating the specificity of the antibodies (Supplementary Figure S2).

When further investigating the expression of p-p21, a possible trend towards a reverse correlation to p21 was observed, however this was not significant (Figure 1G). Comparing the expression of p21 and p-p21 among the tested cell lines, two cell lines, IMR-32 (14.2% ± 6.0 > 2.1% ± 2.2, p<0.05) and MRC5 (45.3% ± 2.7 > 14.0% ± 12.3, p<0.05) had a

lower fraction of p-p21 positive cells, whereas SH-SY5Y (4.7% ± 0.9 < 78.6% ± 17.1, p<0.01), SK-N-FI (10.2% ± 0.9 < 93.7% ± 9.1, p<0.0001), and SK-N-DZ (0.1% ± 0.1 < 97.6% ± 2.2, p<0.0001) had a higher fraction of p-p21 positive cells (Figure 1H).

The protein expression of p21 and p-p21 was also investigated in tumour material from the *TH-MYCN* heterozygote mouse model. The human MYCN cDNA is placed downstream of the tyrosine hydroxylase promoter (Th-MYCN), and the mice spontaneously develop NB at 5.6–19 weeks of age (27, 31). In this material, no cells were detected positive for p21 whereas a significantly higher fraction of tumour cells showed expression of p-p21 (0.0% ± 0.0 < 35.9% ± 16.8, p<0.05) (Figure 1I, J).

Taken together, the RT-qPCR, Western blot, and immunofluorescence data showed that p21 is expressed, in both an un-phosphorylated and a phosphorylated form, heterogeneously across all eight NB cell lines and in the *TH-MYCN* mouse model. MRC5 showed the highest expression of p21 among all the tested cell lines followed by the *TP53* wt cell line SK-N-SH. Moreover, three NB cell lines, SH-SY5Y, SK-N-FI, and SK-N-DZ, and the *TH-MYCN* mouse model showed a preference for p-p21 expression over p21.

Endogenous p21 expression is compatible with replication

Investigation of p21 expression showed positive nuclear staining in a fraction of cells in all the tested NB cell lines (Figure 1). In the nucleus, p21 generally inhibits cyclin-CDK complexes, thus leading to the direct inhibition of cell proliferation (32). However, low expression of p21 has been shown to act as an assembly factor of CDK4/6 and cyclin D thereby aiding initiation of the S-phase (18, 19). Further investigation of the p21 expression in the NB cell lines showed that within the fraction of p21 positive cells, there was heterogeneity in the expression level, with cells expressing low, intermediate, or high levels of p21. Cells with low p21 expression were observed in all the tested cell lines, with SK-N-SH, IMR-32, and SK-N-FI having the highest fraction (8.2% ± 0.9, 8.8% ± 3.6, and 9.3% ± 0.8, respectively). Among the *TP53* wt cell lines, SK-N-SH (8.2% ± 0.9, p<0.01) and IMR-32 (8.8% ± 3.6, p<0.01) showed higher fraction of positive cells compared to SH-SY5Y (2.7% ± 0.4). Among the *TP53* mut cell lines SK-N-FI (9.3% ± 0.8) had the highest fraction of cells expressing low levels of p21 followed by SK-N-AS (5.3% ± 0.5), Kelly (4.8% ± 0.5), BE(2)-C (1.2% ± 0.2), and SK-N-DZ (0.1% ± 0.1) (Figure 2A). Cells with intermediate p21 expression were observed in all cell lines except SK-N-DZ. Moreover the *TP53* wt cell lines, SK-N-SH, IMR-32, and SH-SY5Y had the highest fraction of p21 positive cells with intermediate expression among all the tested cell lines. However, there was no significant difference within either the *TP53* wt cell lines or the *TP53* mut cell lines (Figure 2A). Cells with high p21 expression were observed in all three *TP53* wt cell lines, with SK-N-SH (0.6% ± 0.1, p<0.01) having a significantly higher fraction of positive cells compared to SH-SY5Y (0.2% ± 0.2). There was only one *TP53* mut cell line with cells positive for high p21 expression, SK-N-FI (0.1% ± 0.1). The remaining *TP53* mut cell lines SK-N-AS, Kelly, BE(2)-C, and SK-N-DZ showed no cells with high p21 expression (Figure 2A). Representative picture of low, intermediate, and high p21 expressing SK-N-SH cells are shown in Supplementary Figure S3A.

In order to further investigate whether endogenous p21 expression is compatible with proliferation, a triple staining was performed in all eight cell lines, using antibodies binding to p21, together with a downstream effector of cell cycle arrest, phosphorylated ataxia-telangiectasia mutated (pATM), and the proliferation marker Ki67. First, each marker was analyzed on its own with the fraction of p21 positive cells being higher in *TP53* wt cell lines SK-N-SH and IMR-32, followed by the *TP53* mut cell line SK-N-FI, and lower in the remaining cell lines (Figure 2B), similar to findings in Figure 1. The fraction of pATM positive cells was generally low in all cell lines, except SK-N-FI which had 18.1% ± 25.9 cells positive for pATM. However, there was no significant difference in the fraction of pATM positive cells between the cell lines (Figure 2B). Six cell lines showed high fraction of proliferation, > 60% Ki67 positive cells, consistent with active replication. Two cell lines, SK-N-SH and SK-N-AS had a slightly lower fraction of Ki67 positive cells (34.9% ± 7.1 and 41.4% ± 14.4, respectively) (Figure 2B).

Analysis of co-expression of the markers showed that within each cell line, a very small fraction of p21 expressing cells displayed co-expression of p21 and pATM (0-0.2%) (Figure 2C), confirming that the endogenous p21 expression is primarily not present in response to the DNA damage pathway. Instead, the majority of p21 expressing cells were double positive for Ki67, with the highest fraction of double positive cells observed in SH-SY5Y (95.1%), followed by SK-N-DZ (85.7%), Kelly (65.4%), IMR-32 (65.1%), BE(2)-C (64.3%), and SK-N-FI (62.7%), whereas SK-N-SH (36.4%) and SK-N-AS (25.0%) had the lowest fraction of p21 positive cells which were replicating (Figure 2C). Representative pictures of p21 and Ki67 double positive expressing SK-N-DZ cells are shown in Supplementary Figure S3B.

Overall, these results indicate that in all cell lines, except SK-N-SH and SK-N-AS, the majority of un-phosphorylated p21 expressing cells were associated with Ki67 and not DNA damage, indicating active replication.

Analysis of p21 and p-p21 expression following treatment with cisplatin IC_{50}

In order to investigate a possible role of p21 and p-p21 following cisplatin treatment, the sensitivity of each cell line

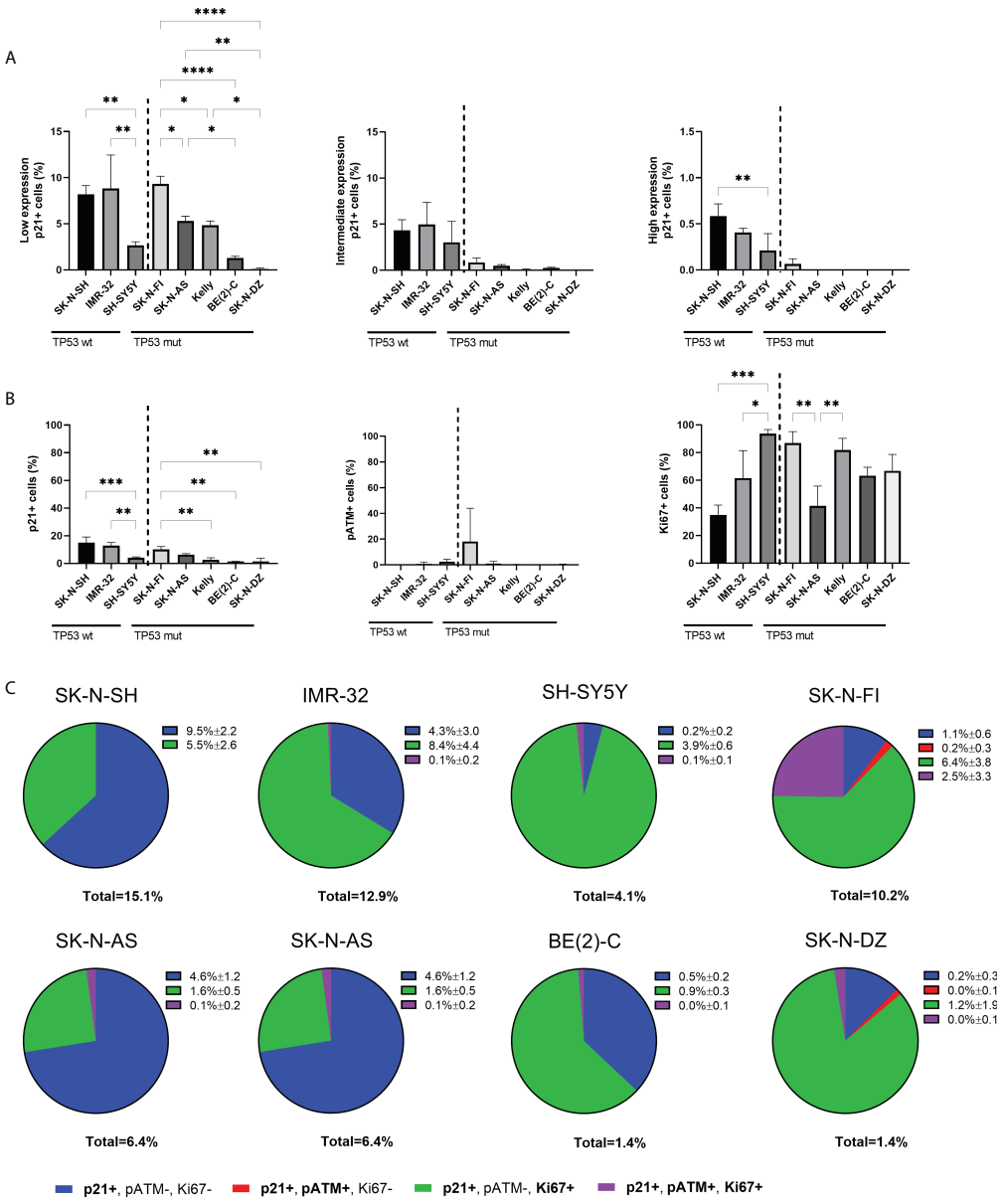


FIGURE 2
Expression levels of p21 and triple immunofluorescence staining with p21, pATM, and Ki67 in NB cell lines. **(A)** Graphs depict percentage of positive cells for low, intermediate, or high endogenous p21 expression. The *TP53* wt cell lines showed cells with expression levels of p21 in all three ranges, whereas only SK-N-FI had expression levels of p21 in all three ranges among the *TP53* mut cell lines. * = p<0.05, ** = p<0.01, **** = p<0.0001, not significant p>0.05. One-way ANOVA with Tukey *post hoc* test. Mean ± SD, n=3. **(B)** Analyzing the fraction of either p21, pATM or Ki67 single positive cells within all cell lines showed overall low fraction of cells positive for either p21 or pATM and a higher fraction of Ki67 expressing cells. * = p<0.05, ** = p<0.01, *** = p<0.001, not significant p>0.05. One-way ANOVA with Tukey *post hoc* test. Mean ± SD, n=3. **(C)** Pie charts of the four different combinations of p21 expressing cells within each cell line, either single p21 positive, p21 and pATM double positive cells, p21 and Ki67 double positive cells or p21, pATM, and Ki67 triple positive cells. The majority of cell lines showed that there are large subpopulations of replicating cells with p21 expression. Mean ± SD, n=3.

towards cisplatin was investigated. Cisplatin showed a concentration-dependent decrease in cell viability after 72 hours of treatment, with IC_{50} values ranging below the tested range for IMR-32 (<0.05 μM) to 32.0 μM for BE(2)-C (Figure 3A). Overall, the *TP53* mut cell lines showed a lower sensitivity compared to the *TP53* wt cell lines. Four *TP53* mut cell lines, SK-N-FI, Kelly, SK-N-DZ, and BE(2)-C, displaying the highest IC_{50} values for cisplatin, were selected for further investigation of the cisplatin response. Each of the four cell lines were treated with the corresponding cisplatin IC_{50}

concentration for 24 hours following analysis, using immunofluorescence, of DNA damage (pATM) and proliferation (Ki67) as a predictor of treatment outcome (33). An increase in the fraction of pATM positive cells was observed in three out of the four tested cell lines (Kelly 3.3% ± 2.5 < 58.3% ± 20.9, p<0.05; SK-N-DZ 0.1% ± 0.1 < 17.5% ± 7.4, p<0.05; BE [2]-C 0.4% ± 0.3 < 18.3% ± 5.2, p<0.01), indicating activation of the DNA damage response pathway (Figure 3B). For one cell line, SK-N-DZ, the fraction of Ki67 positive cells was reduced (72.6% ± 7.5 > 35.4% ± 12.5, p<0.05), whereas a trend in reduction (not significant) was observed for the remaining cell lines (Figure 3B). The reduction in proliferating cells seen in the SK-N-DZ cell line might reflect its relatively short doubling time compared to the other cell lines. Further investigation of p21 and p-p21 expression following cisplatin treatment showed a reduction in the fraction of p21 positive cells in the BE(2)-C cell line (1.1% ± 0.2 > 0.2% ± 0.2, p<0.01), whereas an increase in the fraction of p-p21 positive cells was observed in Kelly (26.6% ± 6.1 < 58.5% ± 9.8, p<0.01) and BE(2)-C (25.9% ± 13.7 < 44.3% ± 7.6, p<0.05) (Figure 3C). When investigating the fraction of positive cells for each marker we observed a change in the intensity of the expression. Therefore, we also measured the mean expression (mean fluorescence in arbitrary units per cell ± SD) of either p21 or p-p21. In the SK-N-DZ cell line there was a reduction in the mean expression of p21 (0.44 ± 0.06 > 0.25 ± 0.04, p<0.05), whereas an increase was observed for p-p21 (2.50 ± 0.11 < 4.89 ± 0.33, p<0.001), indicating a shift from the un-phosphorylated to the phosphorylated protein (Figure 3D). Moreover, Kelly showed a slight but significant reduction in the mean expression of p-p21 following treatment (2.41 ± 0.07 > 2.14 ± 0.15, p<0.05) (Figure 3D).

Taken together, the *TP53* mut cell lines were more resistant to cisplatin treatment compared to the *TP53* wt cell lines. Furthermore, an increase in the fraction of pATM positive cells, indicating activation of the DNA damage pathway, was observed following treatment with cisplatin at the corresponding IC_{50} concentration for three of the four cell lines investigated. Two of the four tested cell lines, SK-N-DZ and BE(2)-C, had a decrease either in the fraction or the mean intensity of p21 positive cells. At the same time, three of the four tested cell lines, all but SK-N-FI, had an increase in either the fraction or the mean intensity of p-p21 positive cells, indicating that there is a preference of p-p21 activation, compared to p21, following cisplatin treatment.

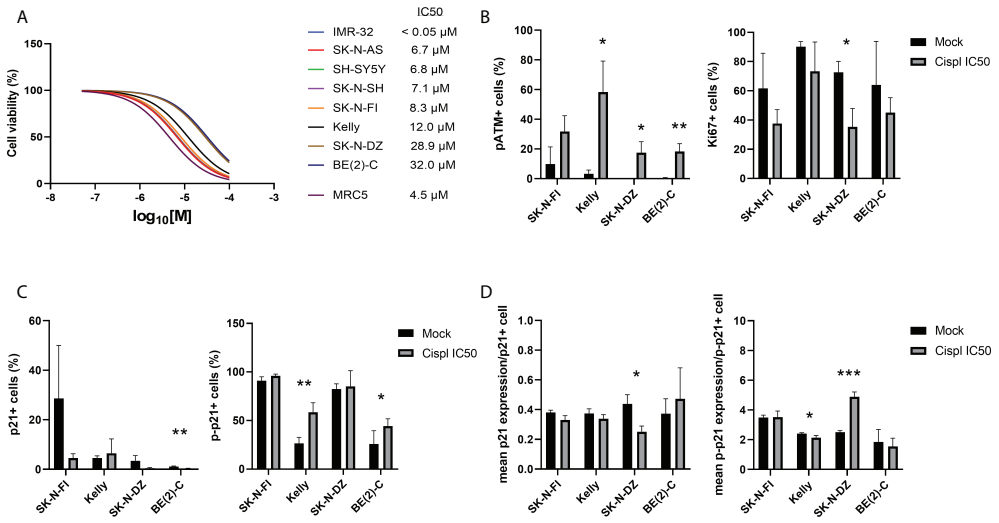


FIGURE 3
Immunofluorescence staining of p21, p-p21, pATM, and Ki67 in NB cell lines following treatment with cisplatin. **(A)** Dose–response curves for cell viability after 72 hours of cisplatin treatment in eight NB cell lines and the fibroblast cell line, MRC5. Cell viability was assessed using MTS-assay to determine the IC_{50} values of cisplatin as a single agent. Concentration ranges, from 100 µM to 0.05 µM of cisplatin, was added. *TP53* wt cell lines were in general more sensitive compared to the *TP53* mut cell lines. n=3. **(B)** Analyzing the fraction of either pATM or Ki67 single positive cells following treatment with cisplatin for 24 hours at the corresponding IC_{50} concentration for each of the cell lines. The fraction of pATM was increased in three out of four cell lines, and SK-N-DZ showed a reduction in the fraction of Ki67 positive cells following treatment. * = p<0.05, ** = p<0.01, not significant p>0.05, Student´s t-test. Mean ± SD, n=3. **(C)** Analyzing the fraction of either p21 or p-p21 single positive cells following treatment with cisplatin for 24 hours at the corresponding IC_{50} concentration for each of the cell lines. BE(2)-C showed a decrease in the fraction of p21 positive cells, whereas Kelly and BE(2)-C showed an increase in the fraction of p-p21 positive cell. * = p<0.05, ** = p<0.01, not significant p>0.05, Student´s t-test. Mean ± SD, n=3-6. **(D)** Analyzing the mean intensity (mean fluorescence in arbitrary units per cell ± SD) of either p21 or p-p21 single positive cells following treatment with cisplatin for 24 hours at the corresponding IC_{50} concentration for each of the cell lines. SK-N-DZ showed a decrease in the mean intensity of p21 positive cells, whereas Kelly showed a decrease in the mean intensity of p-p21 positive cells, and SK-N-DZ showed an increase in the mean intensity of p-p21 positive cell. * = p<0.05, *** = p<0.001, not significant p>0.05. Student´s t-test. Mean ± SD, n=3-6.

Determination of sensitivity to the p21 inhibitor UC2288

Most of the tested NB cell lines showed high endogenous expression of p-p21 and/or an activation of p-p21 following cisplatin treatment. In order to investigate the sensitivity of the NB cell lines to the inhibition of p21 and p-p21, a small molecular inhibitor of p21 namely UC2288 was used. Treatment with UC2288 showed a concentration-dependent decrease in cell viability after 72 hours of treatment, with IC_{50} values ranging between 4.3 µM to 53.9 µM (Figure 4A).

UC2288 is reported to inhibit p21 transcriptionally and/or post-transcriptionally (26), therefore as a proof of concept, inhibition assays were performed on the *TP53* mut cell line Kelly and the *TP53* wt cell line SH-SY5Y. A concentration of 10 µM of UC2288 was selected, since higher concentrations have been reported to give adverse effects (29). Three time point were selected, 2 hours, 4 hours and 6 hours post-treatment, and p21 mRNA and protein levels were assessed using Western blot and immunofluorescence. In the Kelly cell line, a decrease in the p21 mRNA levels were observed after 2 hours treatment followed by increasing levels after 4 hours and 6 hours treatment (Figure 4B), which might suggest stress induction, similar to findings by Taubenberger and colleagues (29). The expression of p21 was not altered when investigated with Western blot, instead UC2288 successfully reduced p-p21 expression following treatment at all three tested time points ($p<0.01$ or $p<0.001$) (Figure 4C). Moreover, when investigated with immunofluorescence treatment with UC2288 successfully reduced the fraction of p21 positive cells following 4 hours treatment (6.3% ± 2.4 > 1.9% ± 0.5, $p<0.05$) and the fraction of p-p21 positive cells following 6 hours treatment (10.0% ± 3.2 > 3.5% ± 1.7, $p<0.05$) (Supplementary Figure S4A). The SH-SY5Y cell line showed an increase in p21 mRNA levels after 4 hours and no change in the fraction of either p21 or p-p21 positive cells at any of the tested time points when investigated using

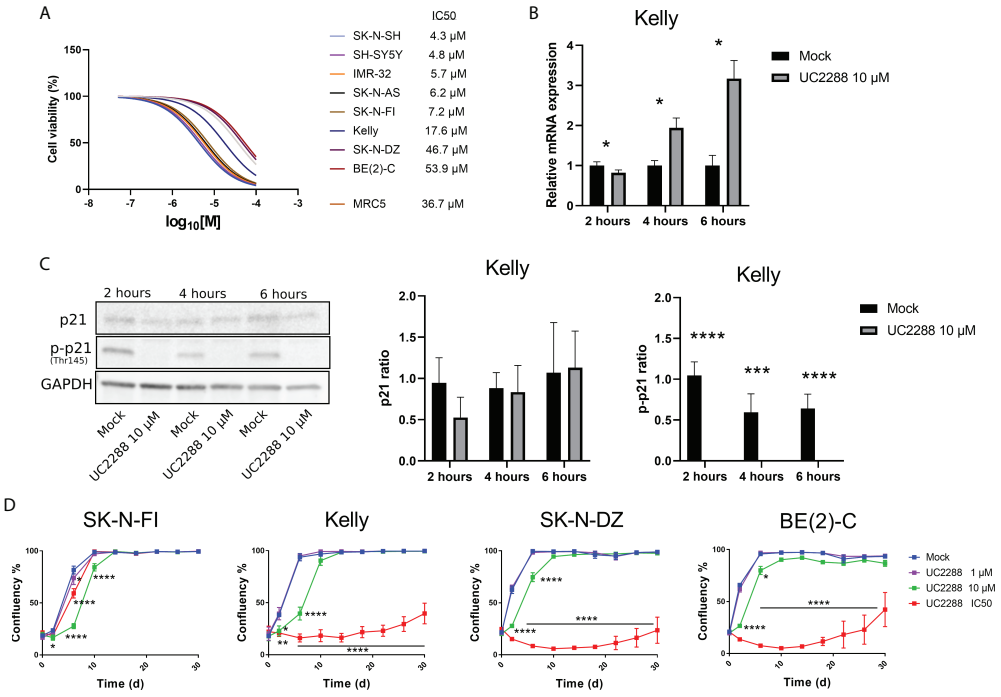


FIGURE 4
Measuring sensitivity to the p21 inhibitor UC2288. **(A)** Dose–response curves for cell viability after 72 hours of UC2288 treatment in eight NB cell lines and the fibroblast cell line, MRC5. Cell viability was assessed using MTS-assay to determine the IC_{50} values of UC2288 as single agents. Concentration ranges from 50 µM to 0.01 µM of UC2288, were added. n=3. **(B)** p21 inhibition assays performed on Kelly with UC2288 at a concentration of 10 µM at three time points. A reduction in mRNA was observed after 2 hours followed by an increase at 4 hours and 6 hours post-treatment. * = $p<0.05$, not significant $p>0.05$, Student´s t-test. Mean ± SD, n=3. **(C)** Western blot analysis showing protein expression of p21 (21 kDa), p-p21 (Thr145) (32 kDa) and loading control GAPDH (37 kDa) in the Kelly cell line following treatment with UC2288 at a concentration of 10 µM at three time points. The expression of p21 was not altered whereas the expression of p-p21 was reduced at all three time points investigated. **** = $p<0.0001$ not significant $p>0.05$, Student´s t-test. Mean ± SD, n=4. **(D)** Cultured cells were exposed to the indicated treatment (mock/UC2288) and analysed using live cell imaging with the IncuCyte at the indicated time points. The confluence shows the cellular densities of each cell line measured over 30 days. The lowest tested dose (1 µM) showed delay in the SK-N-FI cell line. Following either IC_{50} concentration or 10 µM all cell lines showed regrowth capacity, albeit after an extended lag period.* = $p<0.05$, **= $p<0.01$, *** = $p<0.001$, not significant $p>0.05$. Two-way ANOVA with Bonferroni *post hoc* test. Mean ± SEM of 5 experiments.

immunofluorescence (Supplementary Figure S4B). Based on these results, it was confirmed, in the *TP53* mut cell line Kelly, that UC2288 reduces cytoplasmic p-p21 expression.

To determine the long-term effect of UC2288 treatment, regrowth assays were conducted on four *TP53* mut cell lines. Two of these, SK-N-FI and Kelly, showed higher sensitivity to UC2288 compared to the remaining two, SK-N-DZ and BE(2)-C. Regrowth (confluency) was mapped during a period of 30 days following a pulse treatment for 48 hours of mock or UC2288 at three different concentrations: 1 μM, 10 μM or the corresponding IC_{50} value for each cell line. SK-N-FI showed the highest sensitivity to the treatment, with a delay in growth at all three tested concentrations of UC2288 (Figure 4D). Kelly, SK-N-DZ, and BE(2)-C showed a delay in growth at a concentration of 10 μM and a lag period of approximately 26 days following IC_{50} dosing of UC2288 before an increase in confluency (regrowth) was observed (Figure 4D).

Taken together, UC2288 showed a concentration-dependent decrease in cell viability with *TP53* wt cells being more sensitive. UC2288 was able to reduce both the p21 and the p-p21 expression in the *TP53* mut cell line Kelly, and a delay in regrowth was observed in all four tested *TP53* mut cell lines, indicating sensitivity to the drug.

Combination treatment with UC2288 and cisplatin

Investigating a possible correlation in resistance between the single agent UC2288 and cisplatin, showed that cell lines with low IC_{50} values for UC2288 (IMR-32, SK-N-SH, SK-N-AS, SH-SY5Y, SK-N-FI, and Kelly) also had low IC_{50} values for cisplatin. On the other hand, BE(2)-C and SK-N-DZ had higher IC_{50} values for both UC2288 and cisplatin, implying that they are more resistant to single agent treatment with these drugs (Figure 5A). This is supported by the results showing a reverse correlation between the fraction of p21 positive cells and sensitivity to either cisplatin or UC2288 (Figures 5B, C). Based on the correlation in resistance between the single agent UC2288 and cisplatin, we investigated whether a combination treatment of the drugs could be a more effective treatment strategy.

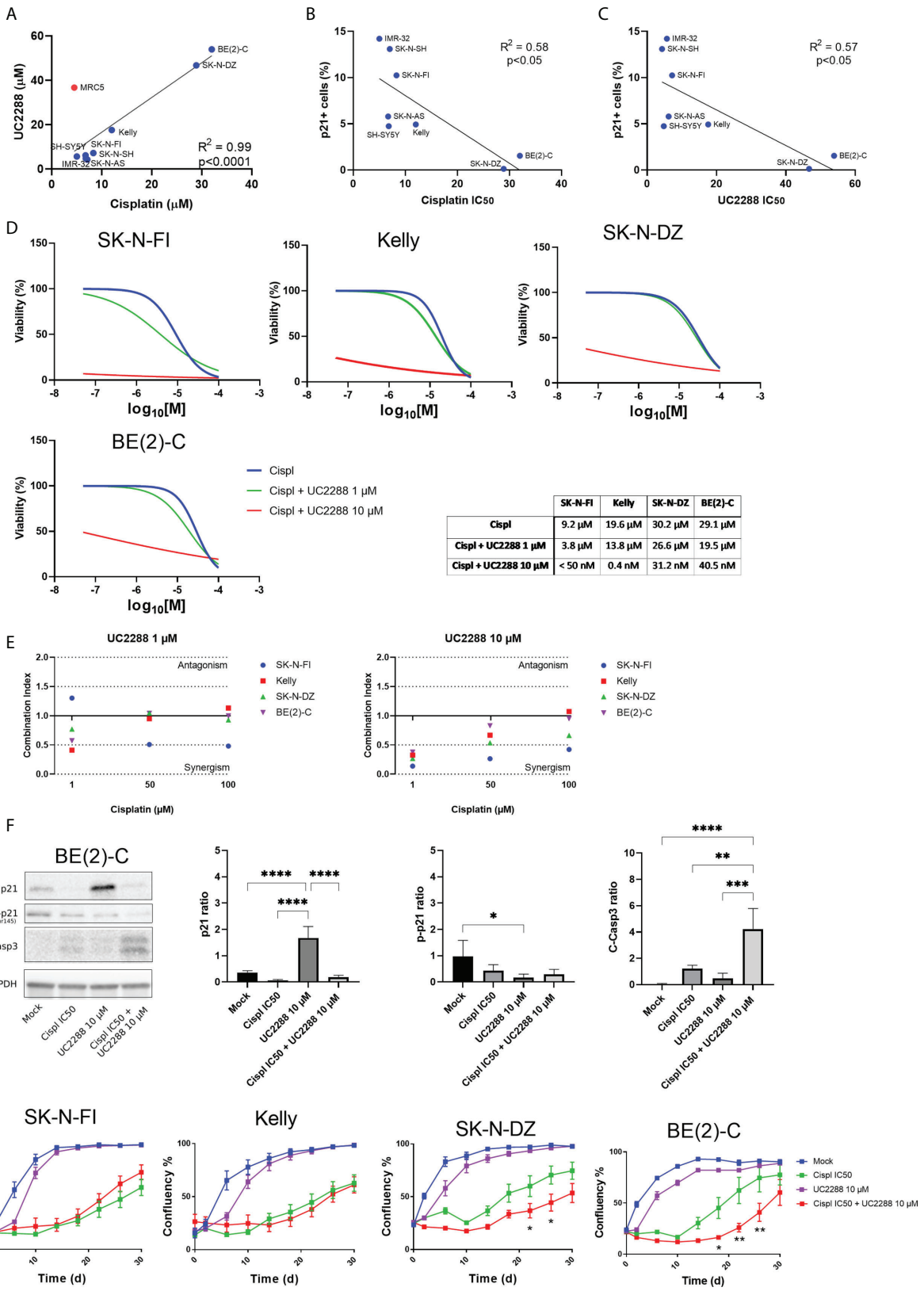
The results showed that combination treatment was effective using UC2288 at a dose of 10 μM for both the more sensitive cell lines, Kelly and SK-N-FI, and the more resistant cell lines, BE(2)-C and SK-N-DZ. Due to the huge shift following combination treatment with UC2288 at 10 μM, the IC_{50} values were calculated based on extrapolated values. The cisplatin IC_{50} value shifted from 19.6 μM to 0.4 nM for Kelly and from 9.2 μM to below range (< 50 nM) for SK-N-FI. For BE(2)-C, the cisplatin IC_{50} value shifted from 29.1 μM to 40.4 nM and for SK-N-DZ from 30.2 μM to 31.2 nM (Figure 5D). To assess whether a shift would be observed with a lower concentration of UC2288, the same experimental setup was used with UC2288 at a concentration of 1 μM. At 1 μM UC2288, a small shift in the cisplatin IC_{50} value was observed, both in the sensitive and in the resistant cell lines (Figure 5D). The combination treatment was also evaluated in the MRC5 cell line, were the cisplatin IC_{50} value shifted from 14.2 μM to below range (< 50 nM) when combined with UC2288 at 10 μM. Moreover, a small shift in the cisplatin IC_{50} value was observed when combined with following UC2288 at 1 μM (Supplementary Figure S5). Combination index analysis was also performed to further evaluate a possible positive synergism, the additive effects or antagonism between cisplatin and UC2288. A synergistic effect was observed in all cell lines following combination treatment with cisplatin with UC2288 at 10 μM and at 1 μM (Figure 5E). The BE(2)-C cell line was further investigated using Western blot and immunofluorescence following treatment with either mock, cisplatin at IC_{50}, UC2288 at 10 μM or a combination of cisplatin IC_{50} and UC2288 10 μM. An increase of p21 was observed with both Western blot and immunofluorescence following UC2288 10 μM treatment (Figure 5F; Supplementary Figure S6). However, whereas a decrease in p-p21 was observed following UC2288 10 μM treatment with Western blot (Figure 5F), this was not observed using immunofluorescence, an increase in the fraction of p-p21 expressing cells was observed following treatment with cisplatin at IC_{50} (Supplementary Figure S6). Moreover, an increase of C-Casp3 following combination treatment was observed with both Western blot and immunofluorescence (Figure 5F; Supplementary Figure S6).

To determine the long-term effect of the combination treatment with UC2288 and cisplatin, regrowth assays were conducted on the four previously selected cell lines. Regrowth (confluency) was mapped during a period of 30 days following a pulse treatment for 48 hours of mock, cisplatin IC_{50}, UC2288 10 μM, or combination of cisplatin IC_{50} and UC2288 10 μM for each corresponding cell line. Two cell lines, SK-N-DZ and BE(2)-C, showed delay in growth following combination treatment compared to a single cisplatin treatment (Figure 5G).

Taken together, combination treatment with UC2288 and cisplatin reduced viability in both sensitive, Kelly and SK-N-FI, and resistant, BE(2)-C and SK-N-DZ, cell lines, even though the effect was more profound in the sensitive cell lines. Furthermore, combination treatment showed increase in cell death, indicated by C-Casp3, and a delay in regrowth compared to a single treatment with cisplatin in the two resistant cell lines, BE(2)-C and SK-N-DZ.

Discussion

The p21 protein is highly versatile, with a multifunctional role as both a tumor suppressor and an oncogene. The functional regulation of p21 relies basically on two post-translational modifications: phosphorylation and ubiquitylation (34), where the process of phosphorylation serves to regulate the p21



	SK-N-FI	Kelly	SK-N-DZ	BE(2)-C
Cispl	9.2 μM	19.6 μM	30.2 μM	29.1 μM
Cispl + UC2288 1 μM	3.8 μM	13.8 μM	26.6 μM	19.5 μM
Cispl + UC2288 10 μM	< 50 nM	0.4 nM	31.2 nM	40.5 nM

FIGURE 5 (Continued)

FIGURE 5 (Continued)
Combination treatment with UC2288 and cisplatin. **(A)** UC2288 versus cisplatin IC_{50} values and the correlation between the two. A high correlation between sensitivity to UC2288 and cisplatin was observed, MRC5 was excluded from the correlation analysis. Overall, SK-N-SH and IMR-32 showed the highest sensitivity to UC2288 and cisplatin whereas SK-N-DZ and BE(2)-C showed most resistance. Linear regression with Pearson's correlation coefficient, p<0.0001. n=3. **(B)** A reverse correlation was observed for the fraction of p21 positive cells and sensitivity to either cisplatin or **(C)** UC2288. Linear regression with Pearson's correlation coefficient, p<0.05. n=3. **(D)** Viability assay on four resistant cell lines, Kelly, SK-N-FI, BE(2)-C, and SK-N-DZ, following treatment with cisplatin as a single reagent or in combination with UC2288 (1 μM or 10 μM). A reduction in IC_{50} was observed in all cell lines following combination treatment compared to a single cisplatin treatment. n=3. **(E)** Combination treatments with cisplatin and UC2288 at either 1 μM or 10 μM. Combination index (CI) analysis was calculated in four NB cell lines, CI<0.7 suggests synergy, CI>1.45 antagonism, and 0.7<CI<1.45 additive combinational effects. **(F)** Western blot analysis showing protein expression of p21 (21 kDa), p-p21 (Thr145) (32 kDa), C-Casp3 (19 kDa and 17 kDa), and loading control GAPDH (37 kDa) in the BE(2)-C cell line following 24 hours treatment with Mock, cisplatin at IC_{50}, UC2288 at 10 μM, or a combination of cisplatin IC_{50} and UC2288 10 μM. An increase was observed of p21 following UC2288 treatment, a decrease in p-p21 following UC2288 treatment and an increase of C-Casp3 following combination treatment. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, not significant p>0.05. One-way ANOVA with Tukey *post hoc* test. Mean ± SD, n=4. **(G)** NB cell lines were treated with cisplatin and/or UC2288 and regrowth was measured over 30 days with an IncuCyte ('confluence' value indicates the cellular densities). The resistant cell lines, BE(2)-C and SK-N-DZ, showed a reduction in growth following combination treatment with cisplatin and UC2288 compared to a single cisplatin treatment. BE(2)-C showed highest sensitivity for combination of the drugs. * = p<0.05, **= p<0.01. Two-way ANOVA with Bonferroni post tests. Mean ± SEM of 5-10 experiments.

activity, localization, stability, and degradation. In this study we show in a panel of eight high-risk NB cell lines, endogenous expression of both un-phosphorylated and phosphorylated p21 (Thr145). The expression of p21 and p-p21 was not dependent on functional p53, since both *TP53* wt and *TP53* mut cell lines showed expression. However, there was discrepancies between the expression level of p21 mRNA and the p21 protein, both as un-phosphorylated and phosphorylated protein. This is in concordance with studies showing general poor correlation between expression levels of mRNA and protein (37, 38), a discrepancy which might be cell-type specific and attributed to other levels of regulation between transcript and protein product (39).

Nevertheless, our data support the notion of p21 as an oncogene in NB. The un-phosphorylated p21 protein was expressed in a small fraction of unstressed proliferating cells, in line with the role of p21 as an assembly factor for CDK4/6 complexes during G1 transition (19). However, despite the possible role of p21 in cell cycle progression other drivers i.e. MYCN, CDK4/6, and cyclins, in cell cycle progression most likely reflect the proliferation status of the cell lines. Indeed, both the cell lines SK-N-SH and SK-N-AS, which showed the least fraction of proliferating p21 positive cells also lack amplification of the proto-oncogene MYCN (40).

Whereas the fraction of p21 expressing cells was low among the tested NB cell lines, a higher fraction of cells showed expression of p-p21, displaying heterogeneity in its cellular localization. All of the tested cell lines, except SK-N-DZ, displayed endogenous cytoplasmic localization of p-p21, suggesting that the Thr145 localization driver may be cell-type specific. Similar results have been observed by others where p-p21 was localized both in the cytoplasm and in the nucleus (41, 42). Furthermore, both cytoplasmic and nuclear p-p21 have been suggested to be pro-tumourigenic, where cytoplasmic p-p21 abrogates or downregulates apoptotic responses (21, 32) and nuclear p-p21 causes loss in its ability to interact with the proliferating cell nuclear antigen (PCNA), thereby facilitating proliferation in endothelial cells (42).

Following investigation of sensitivity to cisplatin treatment, the four cell lines displaying the highest resistance were selected for further analysis. Among the selected cell lines all have *TP53* mut and three, SK-N-DZ, Kelly, and BE(2)-C, have *MYCN* amplification. The clinical prevalence of these genetic alterations is seen among 20-30% of NB tumours which have *MYCN* amplification, and *TP53* mut are observed in a high proportion following relapse, indicating a mechanistic relevance in the development of therapy resistance (43). Morover, the p21 protein, either un-phosphorylated or phosphorylated, is rarely inactive following relapse (35, 36), suggesting it might be an attractive therapeutic target. The fraction or the mean intensity of p21 positive cells was either reduced or unaltered in four of the four tested NB cell lines, indicating that p21 might not be a driver of cell cycle arrest in NB following cisplatin treatment. This is similar to previous findings in NB cell lines treated with low concentrations (<0.2μM) of doxorubicin where no induction of p21 was observed (44). However, higher concentrations of doxorubicin (1 μM) have been shown to induce p21 in NB cell lines (45), suggesting that the induction of p21 might be concentration dependent. Moreover, an increase in either the fraction or the mean intensity of p-p21 positive cells was observed in three of the four tested cell lines, Kelly, SK-N-DZ, and BE(2)-C, indicating a preference for the anti-apoptotic function of p-p21 over its cell cycle regulatory function in NB cells, similar to findings in clear-cell carcinomas, testicular cancer, and ovarian cancer treated with cisplatin (11, 12, 46).

In order to sensitize NB cells to cisplatin we used the p21 inhibitor UC2288. First, we investigated the ability of UC2288 to

inhibit p21 and/or p-p21 in NB cell lines. In the *TP53* mut cell line Kelly there was an initial reduction in the p21 mRNA expression and a transient reduction in the fraction of p21 positive cells following treatment with UC2288. This indicates that the mechanism by which UC2288 attenuates p21 occurs by means of transcriptional or post-transcriptional regulation and not *via* protein degradation (26). Moreover, a significant downregulation of p-p21 was observed at all three tested time points when analyzed with Western blot and following 6 hours treatment when analyzed with immunofluorescence. The difference between the p-p21 data generated by the two methods most likely reflects the sensitivity of the p-p21 antibody for both cytoplasmic and nuclear p-p21 when applied in immunofluorescence. Indeed, UC2288 has been shown to more selectively target cytoplasmic p-p21 compared to nuclear p21 (26). Furthermore, treating the *TP53* wt cell line SH-SY5Y with UC2288 did not reduce the fraction of p21 or p-p21 positive cells. This most likely reflects the cellular localization of the proteins since SH-SY5Y expresses predominantly nuclear p21 and p-p21. Similar results was also seen following treatment with UC2288 in the BE(2)-C cell line where a reduction of cytoplasmic p-p21 was observed when analyzed with Western blot but not when analyzed with immunofluorescence which detects both cytoplasmic and nuclear p-p21 expression, further validating the ability of UC2288 to inhibit mainly cytoplasmic p-p21. Further treatment of *TP53* mut NB cell lines with UC2288 in combination with cisplatin showed a synergistic effect and a dramatically reduced the IC_{50} value, with an increase in cell death, indicated by C-Casp3 activation, suggesting UC2288´s potential as a drug against chemotherapy resistance (47). Moreover, regrowth was delayed in the two most cisplatin-resistant cell lines, SK-N-DZ and BE(2)-C, when UC2288 was given in combination with cisplatin, further indicating the potential of the p21 inhibitor as a drug targeting cisplatin resistant cells.

Overexpression of cytoplasmic p21 is found in a variety of human cancers, including renal cell carcinoma, breast cancer, pancreatic cancer, testicular cancer, ovarian cancer, cervical cancer, squamous cell carcinomas and prostate cancer (47). In many cases, p21 upregulation correlates positively with poor prognosis, tumour grade, invasiveness and drug-resistance (32, 47). However, in patient derived high-risk NB tumours it was suggested that lower levels of p21 expression could be associated with poorer outcome (48). A reverse correlation was also observed for the fraction of p21 positive cells and sensitivity to either cisplatin or UC2288 in our data, supporting the findings seen in patients. Moreover, our data indicate a general reverse correlation between p21 and p-p21 where cells with low p21, and higher p-p21, are more resistant to treatment (Figures 1, 5A-C). It is therefore important to increase our understanding of the paradoxical function of p21 to effectively design therapeutic strategies. This is of interest since treatment with the UC2288 original construct sorafenib demonstrate inhibition of growth of NB tumours by targeting both NB cells and tumour blood vessels (49), whereas it gave minimal anti-tumour activity in NB patients with relapse and refractory NB (50). The discrepancy between primary and relapse NB tumours treated with sorafenib might be explained by aberrations in signaling pathways regulating p21. Cisplatin has been shown to activate PI3K/Akt in several cancer cell lines (51), suggesting that even in NB chemotherapy induced Akt might drive phosphorylation of p21, thereby shifting the balance towards its oncogenic properties (16). Furthermore, aberrant activation of the PI3K/Akt pathway has been shown to correlate with poor outcome in NB (52, 53). Therefore, targeting both the PI3K/Akt pathway and p21 in combination with chemotherapy might give an even more potent effect. This is also supported by findings where inhibition of the PI3K/Akt signaling pathway was suggested to represent a clinically relevant target for the treatment of high-risk NB patients (54).

In conclusion we demonstrate an important mechanism, dependent on p-p21 expression levels in NB, that mediated resistance to cisplatin. Moreover, we provide a target to overcome resistance, thereby our findings might offer an alternative therapeutic strategy in order to reduce side effects and improve treatment outcome.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Stockholm ethics committee for animal research (no. 5163-2019).

Author contributions

SSF contributed to conceptualization, data curation, methodology, project administration, resources, software, supervision, visualization, and funding acquisition. AS, VH and SSF performed the formal analysis, statistical analysis,

investigation, and validation. AS, MW and SSF wrote the first draft of the manuscript. MW and SSF wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This research was funded by the Childhood Cancer Foundation grant numbers, TJ2019-0118, PR2019-0101, and PR2021-0037, the Swedish Cancer Society grant number 21-0313 SIA. Eva and Oscar Ahrén Foundation, Stockholm, and Magnus Bergvalls stiftelse grant number 2021-04262.

Acknowledgments

The authors wish to thank Dr. Per Kogner for NB cell lines and Dr. Mohammad Hojjat Farsangi for the HL-60 cell line. We would also like to thank Dr. Ingrid Lilienthal and Hala Ibrahim Mohammad Habash for scientific discussions and technical assistance.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.906194/full#supplementary-material

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frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 04 October 2022
DOI 10.3389/fonc.2022.945911

Check for updates

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 17 May 2022
ACCEPTED 16 September 2022
PUBLISHED 04 October 2022

CITATION
McLoone JK, Chen W, Wakefield CE, Johnston K, Bell R, Thornton-Benko E, Cohn RJ and Signorelli C (2022) Childhood cancer survivorship care: A qualitative study of healthcare providers' professional preferences. *Front. Oncol.* 12:945911. doi: 10.3389/fonc.2022.945911

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Childhood cancer survivorship care: A qualitative study of healthcare providers' professional preferences

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Purpose: Childhood cancer survivorship care is a complex specialty, though it is increasingly being integrated into the general practitioner's (GP) remit. Establishing the essential components of tertiary- and primary-led care, to maximize the benefits and overcome the challenges inherent to each, is essential to inform the development of survivor-centered, sustainable care models.

Methods: We used the qualitative principles of semi-structured interviewing, verbatim transcription, coding (supported by NVivo12) and thematic analysis, to collect and evaluate the views and preferences of pediatric oncologists, survivorship nurse coordinators, and GPs currently caring for childhood cancer survivors.

Results: Seventy healthcare providers (19 oncology staff and 51 GPs) from 11 tertiary hospitals and 51 primary practices across Australia and New Zealand participated. Participants reported specialist expertise and holistic family-centered care as the key benefits of tertiary and primary care respectively. Participants reported that tertiary-led survivorship care was significantly challenged by a lack of dedicated funding and costs/travel burden incurred by the survivor, whereas primary-led survivorship care was challenged by insufficient GP training and GPs' reliance on oncologist-developed action plans to deliver guideline-based care. GPs also reported a need for ongoing access to survivorship expertise/consultants to support care decisions at critical times. The discharge of survivors into primary care limited late-effects data collection and the rapid implementation of novel research findings.

Conclusions: Healthcare professionals report that while a risk-stratified, collaborative model of survivor-centered care is optimal, to be implemented successfully, greater provisions for the ongoing engagement of GPs and further access to GP education/training are needed.

KEYWORDS
Models of care, pediatric, pediatric oncology, survivorship, primary care, shared care

Introduction

Providing long-term, comprehensive cancer survivorship care to all childhood cancer survivors (CCS) is extremely Important given their lifelong risk of developing complex, chronic, and comorbid health concerns, including but not limited to cardiovascular, endocrine, and reproductive concerns, concerns related to the central nervous system such as learning disorders and epilepsy, as well as social, functional, and mental health concerns (1). While advancements are being made in the complex treatment of children with cancer (e.g. rapid advances in precision medicine (2, 3) and immunotherapy (4)) and services (e.g. telehealth), the challenge to provide optimal care continues to increase as more patients are surviving cancer and living longer lives and the high-needs CCS population continues to grow (5, 6). Particular complexity relates to providing more accessible and engaging care for the 68-81% of survivors who do not currently participate in long term follow-up care, due to personal barriers (such as travel costs, medical anxiety, or time constraints), systemic barriers (such as poor access and few survivorship clinics nationally), and age-related restrictions that limit access to their treating team once they have reached adulthood (7–9) A recent review of global childhood cancer survivorship care pathways indicates that almost all countries have difficulty managing this transition period and providing standardized care into adulthood (5).

Since the seminal report, "From cancer patient to cancer survivor: Lost in transition" was published in 2006 (10), greater research and resources have been focused on developing an optimal model of survivorship care (11). Almost 15 years later, there is a continued acknowledgement that there is unlikely to be one single, ideal model, as clinicians and researchers recognize that one size does not fit all - all survivors and all local settings have inherently varied resources and limitations. Many national cancer bodies now recommend risk-stratified pathways, involving oncologist and general practitioner (GP) involvement to varying degrees, as determined by the survivor's age, the cancer treatment they received, their risk of recurrence, chronic health conditions, supportive care needs, and circumstances (12).

Within childhood cancer survivorship, models of care are not usually disease specific, but rather focus on providing late-effects expertise *via* a tertiary care model (including oncologist-led, nurse-led, or multidisciplinary models of care), a primary care model (GP-led), or a shared care model (which envisages both tertiary and primary clinical teams working collaboratively) (13). The Journal of Cancer Survivorship recently called for further research focused on tailored models of care to elucidate exactly who, where, and for which survivors particular models are optimal, acknowledging that it is unlikely for comprehensive cancer care to be delivered by one type of specialist exclusively (14).

Given that oncologists, nurse specialists, and GPs all play pivotal roles in the provision of survivorship care, we sought to better understand their views on hospital-based, GP-based, and shared-care models. We sought to explore health professionals' views on the limitations of these models in their current form, as well as what they consider to be critical to the successful implementation of feasible, sustainable, engaging and equitable life-long survivorship care.

Materials and methods

The Australian and New Zealand Children's Hematology and Oncology Group (ANZCHOG) Survivorship Study (15) surveyed childhood cancer survivors and oncology staff from every tertiary, paediatric oncology treatment centre in Australia and New Zealand (representing 11 children's hospital). Oncology staff were invited to participate if they were the Head oncologist or the clinical nurse consultant (or equivalent) of the survivorship clinic within their hospital. Survivorship oncologists and nurse specialists were invited to participate in this study *via* a mailed letter and follow-up phone call. Survivors participating in the ANZCHOG Survivorship Study were also asked to nominate their current GP, who was then contacted by the research team *via* a mailed invitation letter, and invited to participate in the study.

A multidisciplinary team, including a clinical oncologist, psychologist, survivorship nurse, behavioural scientist, GP, and hospital director, developed two semi-structured interview guides. One guide was to interview oncology staff and the other to interview GPs. The oncology staff interview explored survivorship care practices within the hospital setting (e.g. clinic information, eligibility, guideline adherence), transition pathways, views on models of care, resource needs, challenges and potential solutions. The GP interview explored survivorship care practices within the primary care setting (e.g. GPs' confidence, knowledge, and communication with oncology teams), and GPs' views on models of care, resource needs, challenges and potential solutions. GP demographic data,

including gender, age, years of practice, number of survivors in their practice and postcode of their practice location was collected during the interview.

First author JM (PhD) has over a decade in qualitative research practice and conducted all oncology staff interviews, while a PhD qualified research assistant completed the GP interviews. JM was known to some oncology staff, as an active member within the Australian paediatric oncology survivorship community. The interviewer had no relationship to GP participants. Interviews were conducted in private, were audio recorded, field notes were made and recordings were transcribed verbatim. Analysis was guided by the Braun and Clarke approach (16). Authors JM and WC read all interviews for familiarity with their content. The coding heirarchy was developed based on these initial readings. Interview data were then coded line by line and preliminary themes were organised to establish central core ideas. The South-Eastern Sydney Local Health District granted ethical approval (Reference: 12/173).

Results

We interviewed the lead pediatric oncologist at each of the ANZ tertiary survivorship clinics (n=9) and their lead survivorship nurse (typically a clinical nurse consultant) (n=10) (noting that two oncologists led two clinics each and one nurse led two clinics). Fourteen participants worked in an Australian hospital and five in a New Zealand hospital, average interview length was 34 minutes. Fifty-one GPs were also interviewed, average interview length was 19 minutes. See Table 1 for participants' demographic details. Of the 19 invited oncology staff, 19 participated (100% response rate); out of the 160 invited Australian GPs, 74 opted to participate (46% response rate), but data saturation was achieved after 51 interviews, so no further interviews were scheduled. We considered data saturation to have been achieved at the time when subsequent interviews were no longer contributing additional or new information.

Several themes were identified during analysis of the HCP interviews. These central themes are reported below.

The importance of expert multidisciplinary care

Oncologists and GPs shared their ongoing support for traditional, hospital-based case management with multidisciplinary team (MDT) involvement, as they believed this offered the highest level of expertise and knowledge available to survivors. "[Hospital-based care offers] *particular knowledge and expertise in post-cancer treatment and late effects*" (male, oncologist). Participants reported that oncologists, with survivorship expertise, were more likely to rapidly incorporate new research findings and advances in the field into the care they provided. They reported that oncologist specialists were often more aware of the broad ranging issues survivors faced, such as mental health concerns, financial toxicity, and educational or career impacts, and offered clear pathways to relevant support networks available within the survivor's community.

Hospital-based, MDT care was also viewed as a convenient "*one stop shop*" where survivors could receive a holistic review and care for many of their comorbidities concurrently. Oncology staff reported preferences for greater involvement of multidisciplinary medical specialists (e.g. neurology, adolescent medicine, onco-fertility) and allied health professionals (e.g. dietitians, physiotherapists, disability service coordinators) within their survivorship clinics. However, while more staff were desired, oncology staff expressed their difficulty in securing enough protected time even for their own staff to contribute to survivorship clinics, over and above their clinical roles.

"…lack of funding, lack of resources, lack of space … lack of staff … lack of protected time … there's only a finite number of people we can see each year." (female, clinical nurse consultant (CNC))

Managing survivors' unique needs over time

Despite strong support for expert, oncology-led care with involvement from a broad range of specialties, it was also widely acknowledged that survivors had unique risk profiles, surveillance, and care needs and that this changed over the course of the survivorship period.

In terms of unique risk profiles and needs, participants highlighted the importance of risk-stratification to improve the efficacy of survivorship care delivery.

"You could definitely have triage clinics to determine who would go where, you could base a lot of it on diagnostic risk stratification" (female, CNC)

In terms of survivors' unique needs over time, it was suggested that the existing adult survivorship care infrastructure could be leveraged to also accommodate adult survivors of childhood cancer.

"…as survivorship develops as a significant entity amongst adult patients … there may be an opportunity to obtain synergy with adult clinics with better understanding of the issues of long-term survivors." (male, oncologist)

However, while transitioning pediatric survivors to adult survivorship clinics was viewed as a possible solution in the longer term, there remained a current lack of transition services, causing a reluctance to discharge survivors as "*there's nowhere to put them.*" (female, CNC)

TABLE 1 Demographic information of GPs and oncology staff.

Demographics	GPs (n=51)	Oncology staff (n=19)
Female	22 (43.1%)	13 (68.4%)
Mean years of practice (SD)	28.25 (12.2)	N/A
Mean number of CCSs cared for (SD)	2.16 (1.73)	N/A
Country of practice		
Australia	51	14
New Zealand	0	5
ARIA Class of practice		
Major city	28	19
Inner regional	9	0
Outer regional	2	0

CCS, childhood cancer survivor; ARIA, accessibility/remoteness index of Australia calculated using practice postcodes (17), the distribution of respondents is reflective of the geographic distribution of GPs practicing in Australia; GP, general practitioner; SD, standard deviation; N/A, not assessed.

"There's reluctance [for] *some adult facilities to take* [childhood cancer survivors] *on, there's a lack of clarity about where's the best place to send them."* (female, CNC)

Bridging the gap between tertiary and primary care

There was a general consensus that either an experienced survivorship nurse, or a community GP, could manage low-to-medium risk survivors' care *"…with feedback to late effects clinics in critical times"* (male, oncologist). A large proportion of oncology staff acknowledged the potential benefits of nurse-led survivorship care, with survivorship nurses coordinating survivors' care by liaising with a multidisciplinary team and the survivors' nominated GP.

"From a funding … and logistical perspective, I like the idea of the nurse-led clinic with that person liaising with … community based, or hospital based [providers]." (female, oncologist)

Oncologists unanimously acknowledged the importance of involving GPs in follow-up care to *"function as the liaison for the transition out into the community"* (male, oncologist), while emphasizing that *"the treating center … would always have to have some level of involvement"* (female, CNC).

Oncology staff recognized that a shared-care model between hospital and primary care practices required improved communication and closure of the "feedback loop" between specialists and GPs. Oncologists continued to want to receive progress and updates on survivors' health outcomes and their recommended surveillance schedules from GPs. Similarly, GPs wanted greater involvement throughout the course of their patient's cancer treatment so that they were in a well-informed position to continue care during survivorship. Oncology staff perceived that GPs struggled with this lack of continuity, *"…we've taken over the care of the patient and families for … years and then we go 'alright we're done with you, back to the GP'…* [but] *communication in the interim is not always ideal, families move."* (female, CNC). Lack of communication and collaboration with treating teams during treatment left GPs feeling that they lacked insight into their patient's cancer care and how best to continue to deliver care in a collaborative manner.

"I don't have any direct contact with the hospital. I don't know how it works … I don't know any of the team … That's a huge disadvantage." (male, GP, practicing 27 years)

Barriers to improved bi-directional inter-practice communication included a lack of administrative staff to manage scheduling and correspondence, as well as data managers to oversee record-keeping between sites. Survivorship care plans were reported as labor intensive and their dissemination was *"dependent on workload"* (female, oncologist). The importance of the survivorship nurse role in potentially coordinating much of this work was discussed.

"If you've got a specialized nurse that's keeping an eye on everything and … on the communication between specialists, GPs, the nurses and the patient, that'd be very good…" (female, GP, practicing 10 years)

The perceived benefits of receiving care in the community

Though hospital-community collaboration was viewed as challenging, receiving local, GP-led care was seen to overcome the many logistical and financial issues families faced when trying to access tertiary-led care, which is only located in major urban cities.

"When the family goes up to Sydney … it costs a fortune to stay up there and [they] *can't afford it"* (male, GP, practicing 39 years),

Some GPs felt confident that they could lead survivors' care, noting that their strong counselling practices were ideal for providing *"patient-centered care"* (female, GP, practicing 35 years) and mental health support. Additionally, they reported that the *"traditional family model"* (male, GP, practicing 43

years) within GP services provided a sense of long-term continuity and the opportunity for whole-family care, noting the impact of childhood cancer on the whole family.

"Patients are very familiar with [long-term practice managers] *… it's not uncommon for patients to come back after years …* [we] *pull up the old records and roughly know what it's going to be about* [and] *just book them in…"* (male, GP, practicing 43 years)

The perceived challenges of receiving care in the community

Both oncology staff and many GPs reported low confidence in local GPs' knowledge of survivorship care, as childhood cancer *"is a relatively rare condition"* and there is a *"big lack of knowledge and education"* (female, GP, practicing 14 years) surrounding survivorship issues within the primary sector. GPs also perceived survivors' lack of confidence and trust in their care.

"I don't think that they [the survivor] would have a huge degree of trust - nor would I blame them - in the quality of care offered by the GP." (male, GP, practicing 27 years)

As such, GPs were mostly willing to provide survivorship care for low-risk survivors, with higher risk cases and concerns directed to specialists.

"When these children have a high intensity illness, families get very reliant on [and] *comfortable with the treating specialist. So sometimes their questions are satisfactorily answered at that level."* (male, GP, practicing 25 years)

GPs noted that survivors more often sought medical assistance from them regarding more routine adult health-related issues and that this led to skewed perceptions that survivors have limited risk of developing late-effects. In addition, GPs reported that the large patient volumes seen by GP services acted as a competing interest to furthering childhood cancer survivorship care education, which constituted only a very small proportion of their case load.

"When you're seeing 200 patients a week, I only have enough time and resources to react to what's coming in through the front door." (male, GP, practicing 27 years).

"…the average GP in their lifetime might [probably] *look after one or two* [childhood cancer survivors] *… It's not something that's top on our list of getting really good at"* (male, GP, practicing 32 years).

GPs reported that, as appropriate remuneration to provide comprehensive survivorship care within their practice was not available, this limited any economic justification for up-skilling.

"You're asking people who are basically in semi-sweatshop conditions to do a high-level function when neither the patient nor the community want to fund that." (male, GP, practicing 30 years)

Consequently, GPs reported that they often took a more reactive rather than proactive approach to late effects surveillance and highlighted GPs' need for treatment-specific details, as well as *"evidence-based and guideline-driven"* (female, GP, practicing 14 years) surveillance recommendations, from oncologists at the beginning of the treatment phase.

"In the actual stage of the disease … the specialist should pass on as much information as possible [to GPs], *for then* [GPs] *will know what to expect 10 years, 20 years down the line…"* (male, GP, practicing 21 years)

The need for expert recommendations to guide general practitioners

Continuing onward from the treatment stage, GPs noted a prescriptive approach during survivorship was crucial to accurately counsel survivors, clarify responsibilities, and review and formulate management plans. GPs emphasized that information and communication should be succinct, with specific action plans.

"[GPs] *are inundated with information from all quarters … when I get any discharge summary … I only focus on*, [the section] *'GP to action'."* (male, GP, practicing 27 years)

In addition, clear contact information, referral pathways, and the ongoing availability of oncology staff was also reported as crucial for resolving queries and making referrals back to hospitals.

"[This] *allows me to ring them up … if I have a detailed question. Then if I need to refer him back, it's not … out of the blue.…I think it does provide a valuable support for* [GPs] *definitely."* (male, GP, practicing 12 years)

Future improvements

Overall, health professionals acknowledged that survivorship care is *"so variable, depending on individual cases"* (female, GP, practicing 12 years), suggesting that future improvements in survivorship care should allow care to be tailored according to survivors' individual needs rather than being standardized.

"I don't think it would be very helpful to have just a blanket thing that you do for all of them, because you'd be wasting their time." (female, GP, practicing 21 years)

GPs suggested creating a specific Medicare item number (i.e. a specific, billable Medicare service subsidized by the Australian government) for childhood cancer survivors to encourage more GP services to provide higher quality survivorship care.

"[Survivorship care] could actually be time-consuming … GPs are paid on a person-in-the-room basis … if [the extra time] *was remunerated then you'd certainly get a much better uptake in the community of that role."* (female, GP, practicing 8 years)

Australian participants supported the creation of a national database to hold survivor information (for example, diagnostic, treatment, and late effects data). In NZ, where a national database already exists, participants felt that it was not being used to its full potential to determine the prevalence and risk factors for later disease and morbidity, especially among high-risk and rare childhood cancers.

"The database that we're using is national, the format of the health passport is national. Where we haven't progressed further is with doing and looking at what we've found and that's an another something we need money for, we need time, because you know we've [only] got impressions [not evidence]." (female, oncologist)

Further national collaboration was reported as having the potential to support and guide the standardization of care guidelines and survivorship care plans. Oncology staff also noted the benefits of using nationally collated information to develop centralized education materials for GPs and survivors across sites. Additionally, they suggested a more collaborative approach in survivorship research could improve the representativeness of the sample, prevent research replication and overlap, and maximize the use of funding.

"…people [need] *to collaborate to develop databases to ensure that any research coming out of Australia represents a national database and is not just single or a couple of hospitals collaborating…"* (male, oncologist)

Discussion

Oncology staff and GPs were generally aligned in their perceptions of the various benefits and challenges of delivering high quality childhood cancer survivorship care. The time and cost it takes to deliver complex care, encompassing risk-based surveillance, the management of late-effect comorbidities, and the recognized psychosocial impacts associated with a diagnosis of childhood cancer, were perceived as extremely challenging or unsustainable. Risk-stratified care was seen as ideal, with both oncology and general practices collaborating to varying degrees as the survivor traversed the survivorship period. Yet this model was challenged by the high level of communication, instruction, and data sharing that this would require between multiple practitioners, across several decades of survivorship. Furthermore, any form of shared care, or risk-stratified discharge of low-medium risk survivors to primary care would require the provision of greater training for GPs, who consistently report within this study and elsewhere in the literature (18, 19), low confidence to provide up-to-date care for childhood cancer survivors (20). Given that GPs in this study reported caring, on average, for two survivors across a career of 28 years, it is understandable that they may lack the experience and motivation to invest time in training in this field. Greater liaison with, and education delivered by survivorship nurses, or the use of new innovations in training are possible solutions. GP training should be targeted at GPs currently caring for survivors and new research is needed to develop and evaluate high quality, purpose-built, online, brief, content dense eLearning videos, developed specifically for busy GPs.

The potential for specialist nurses to act as liaisons, bridging the gap, coordinating services, facilitating transitions and educating GPs was proffered in this study and has been reported in the previous literature (21). There is likely room for substantial growth in this area and the potential for experienced nurses to lead the development of resource-limited clinics is strong (20). Funding and investment in the next generation of expert nurses to support such positions remains critical, as does further research to evaluate the efficacy of such models of care.

The issue of insufficient funding has been consistently reported as a barrier in coordinating survivorship care in this study and in existing literature (6, 22, 23). Various strategies were offered, including increasing the responsibility of lower-cost nurses (comparative to higher-cost oncologists or a full multi-disciplinary team), and the creation of specific Medicare Benefit Schedule item numbers (funded by the Federal Government) that would allow hospitals and GPs to claim greater funding/remuneration. Despite the varying healthcare billing systems and practices internationally, the aforementioned strategies could be implemented and modified according to locally specific remuneration issues.

Survivorship care plans (which provide information about a survivor's cancer treatment, follow-up care needs, surveillance schedules and health education) were also discussed as a means to increase communication between oncologists and GPs and to provide GPs with specialist recommendations that could be implemented at the local level, reiterating existing literature (24, 25). However, survivorship care plans have demonstrated variable efficacy to date and limited increases in adherence to surveillance recommendations, detection of late effects and prompt referral (13, 26, 27). While our study reports that GPs would prefer that oncologists provide highly prescriptive care plans early on, most GPs typically report not receiving a care plan (25), and oncologists recognized that these were disseminated only as resources allowed, with issues surrounding the labor intensity of preparing survivorship care plans.

It is important to continue to reassess old challenges in light of new solutions. Post COVID-19, a more general reimagining of care delivery, more reliant on telehealth seems possible (28). Distance-delivered care pathways and survivorship care plans can now be supported by smart platforms, computerized algorithms and new technology, reducing some of the time/cost limitations that have been traditional barriers (29) (30, 31). There is strong potential to develop more cost efficient and more equitable care solutions, especially for regional and rural families *via* evolving telehealth or e-health programs. Future research is needed to provide accurate effectiveness and cost data, including costs to the survivor, provider and health system. The feasibility and acceptability of distance-delivered interventions to support

survivorship care has been reported (32), but further evaluation of larger trials, with long-term outcome data is called for.

Study limitations

Participants in our study were selected for their expertise and prior experience in childhood cancer survivorship care and therefore may not represent the views of all health professionals within the field. Despite potential contextual differences between various countries, our findings surrounding providers' preferences are consistent with internationally reported research findings (5) and can be used to supplement and inform future strategies to enhance the delivery of childhood cancer survivorship care globally. There remains an ongoing need for longitudinal studies evaluating changes in providers' preferences over time to keep pace with advancements in the medical field and rapidly changing circumstances within healthcare systems and technology.

Conclusion

The optimal delivery of sustainable care requires input from multiple health professionals and is unlikely to remain static across the entire survivorship period. As such, communication between key providers including oncologists, survivorship nurses, and GPs, needs to be maintained from diagnosis to end of life. Investment in strategies to support collaboration is necessary or else risk-stratified care will remain impeded. Funding for roles to manage communication, points of transition, effective survivorship care plan dissemination and data sharing is required, as is further research to develop and evaluate such initiatives.

Data availability statement

The datasets presented in this article are not readily available to protect participant and patient confidentiality. Requests to access the datasets should be directed to the corresponding author and will only be shared in accordance with the ethical consent provided by participants on the use of confidential/identifiable human data. Requests to access the datasets should be directed to j.mcloone@unsw.edu.au.

Ethics statement

The studies involving human participants were reviewed and approved by the South Eastern Sydney Local Health District. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JM (first author) contributed to the study conception and design, recruitment, interviewing, analysis and manuscript writing. WC contributed to data analysis and manuscript writing. CW contributed to the study conception and design, and manuscript revision, read and approved the submitted version. KJ contributed to design and contributed to manuscript revision, and read and approved the submitted version. RB contributed to design and contributed to manuscript revision, and read and approved the submitted version. ET-B contributed to design and contributed to manuscript revision, and read and approved the submitted version. RC, contributed to the study conception and design and manuscript revision, and read and approved the submitted version. CS contributed to the study conception, recruitment and design and manuscript revision, and read and approved the submitted version.

Funding

The Behavioural Sciences Unit (BSU) is proudly supported by the Kids with Cancer Foundation. JM is supported by the Medical Research Future Fund, Australian Brain Cancer Mission, Brain Cancer Survivorship Grant (MRFBC000002). CW is supported by the National Health and Medical Research Council of Australia (APP1143767 and RG2008300). CS is supported by a Cancer Institute NSW Early Career Research Fellowship (2020/ECF1144). This work has been supported by the Kids Cancer Alliance and a Medical Research Future Fund (MRFF) Brain Cancer Survivorship grant (MRFBC000002).

Acknowledgments

We would like to thank Joseph Alchin for research assistance.

Conflict of interest

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

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frontiers | Frontiers in Oncology

TYPE Mini Review
PUBLISHED 02 November 2022
DOI 10.3389/fonc.2022.963780

Check for updates

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 07 June 2022
ACCEPTED 17 October 2022
PUBLISHED 02 November 2022

CITATION
Cruz-Gálvez CC, Ordaz-Favila JC, Villar-Calvo VM, Cancino-Marentes ME and Bosch-Canto V (2022) Retinoblastoma: Review and new insights.
Front. Oncol. 12:963780.
doi: 10.3389/fonc.2022.963780

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Retinoblastoma: Review and new insights

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Retinoblastoma (Rb), the most frequent malignant intraocular tumor in childhood, is caused by mutations in the retinoblastoma gene (*RB1*) situated on chromosome 13q14.2. The incidence of retinoblastoma is approximately 1 in 17,000 live births with approximately 8,000 new cases diagnosed each year worldwide. Rb is the prototypical hereditary cancer in humans. Autosomal dominant inheritance is seen in 30-40% of cases whereas the non-inherited sporadic type accounts for the remaining 60-70%. Rb arises due to inactivation of both alleles of the *Rb* tumor suppressor gene, which results in a defective Rb protein (pRB) with subsequent cell cycle impairment and uncontrolled cell proliferation. Patients with Rb have survival rates higher than 95-98% in industrialized countries but mortality remains high in developing countries. For example, the mortality rate in Africa is 70%. In all cases of intraocular and extraocular retinoblastoma, there is a need for new therapies that are more effective and carry less risk of toxicity. The Bruckner test is a practical and easy test for the detection of Rb, this test consists of assessing the fundus reflex through the pupil (red reflex) in both eyes simultaneously with a bright coaxial light produced with the direct ophthalmoscope. Rb can be detected by the Bruckner test showing a pupil that shines white or "Leukocoria". Although the diagnosis of Rb remains essentially clinical, the newly identified biomarkers could contribute to early molecular detection, timely detection of micrometastases and establish new therapeutic options for Rb.

KEYWORDS

retinoblastoma, intraocular tumor, leukocoria, children, ocular oncology

Introduction

Retinoblastoma (Rb), the most frequent malignant intraocular tumor in childhood (Figure 1), is caused by mutations in the retinoblastoma gene (*RB1*) situated on chromosome 13q14.2 (1, 2). The incidence of retinoblastoma is approximately 1 in 17,000 live births (3) with approximately 8,000 new cases diagnosed each year worldwide (3).

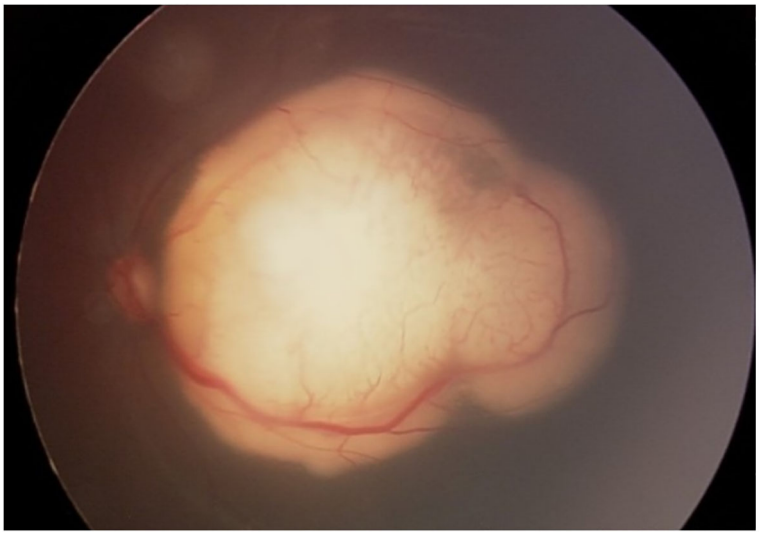

FIGURE 1
Retinoblastoma in the posterior pole.

Rb is the prototypical hereditary cancer in humans. Autosomal dominant inheritance is seen in 30-40% of cases whereas the non-inherited sporadic type accounts for the remaining 60-70% (4). Rb arises due to inactivation of both alleles of the *Rb* tumor suppressor gene, which results in a defective Rb protein (pRb) with subsequent cell cycle impairment and uncontrolled cell proliferation (4).

The *RB1* gene consists of 27 exons that span 183 kilobases (kb) of genomic DNA (4) and encodes a 928 amino-acid nuclear phosphoprotein, pRb (5). pRb is a ubiquitous cell cycle regulator whose activity depends on the level of phosphorylation (5).

The hypophosphorylated form pRb arrests the cell cycle at the G1 restriction point by binding E2F transcription factors (5), which are essential for the expression of genes involved in cell cycle continuity. In Rb, the pRb is functionally inactive due to mutations or deletions (6).

pRb defective or silenced by oncoproteins produced by tumor-causing viruses (SV40, adenovirus, human papillomavirus) prevents exit from the cell cycle and apoptosis and ultimately results in uncontrolled cell division, a hallmark of cancer (1).

Genomic changes in retinoblastoma

Amplification of the *MYCN* oncogene might initiate Rb even in absence of *RB1* mutations. These unilateral RB1 (+/+) MYCN (A) tumors are characterized by distinct histological features and a very early age at diagnosis (7). Other recurring genomic changes that occur in a small minority of tumors include *BCOR* mutation/deletion and *OTX2* amplification (8).

p53 pathway and retinoblastoma

The Rb surveillance pathway mediated by Arf, MDM2, MDMX, and p53 proteins is activated after loss of *RB1* during retinogenesis (8). Subsequent amplification of the *MDMX* gene and increased expression of MDMX protein are strongly selected during tumor progression as a mechanism to suppress the p53 response in *RB1*-deficient retinal cells. The p53 pathway is inactivated in Rb, this cancer does not originate from intrinsically death-resistant cells, as previously thought (9).

Clinical features of retinoblastoma

The most common signs of Rb are leukocoria and strabismus (1). Leukocoria is the presenting sign in 60% of cases (2). The initial presentation is an alteration in the red reflex that, unfortunately, goes unnoticed. So, the diagnosis is usually made in advanced stages and is associated with a worse prognosis. The second most common early sign of retinoblastoma is strabismus, generally related to a macular tumor (2). Less common clinical presentations may also be observed, generally indicating advanced forms (buphthalmos, neovascular glaucoma or orbital inflammation) (2).

10.3389/fonc.2022.963780

Tumor patterns

Typical tumor patterns

- Endophytic: tumor grows into the vitreous and reflects cell proliferation of the internal retinal layers (10).
- Exophytic: tumor develops beneath the retina in the subretinal space and causes overlying retinal detachment (10).
- Diffuse infiltrating: retinoblastoma develops in a flat pattern on the surface of, or beneath, the retina, with no obvious mass, no calcifications, and slow progress (10).

Atypical tumor variants

Cavitary retinoblastoma: tumor contains cavitary spaces, loss of subretinal fluid, and seeding (4).

Anterior retinoblastoma: tumor involves the anterior chamber (4). For some authors, this form of presentation corresponds to the diffuse infiltrating pattern, where a small primary tumor arises in the peripheral retina and then seeds the anterior chamber *via* the aqueous (1, 10).

Retinocytoma: this benign variant may clinically appear as regressed retinoblastoma with associated calcification (1).

Classification of retinoblastoma

The International Intraocular Retinoblastoma Classification (IIRC) is the current guide for Rb staging. Itwas developed by a team of retinoblastoma experts in Paris in 2003 (4, 11).

The IIRC has 5 categories

Group A: Small tumors, 3mm or smaller in their greatest dimension, confined the retina. Located > 3 mm from the fovea and 1.5 mm from the optic disc.

Group B: Tumors greater than 3 mm, located 3 mm or less from the fovea and less than 1.5 mm from the optic disc or that presents subretinal fluid whose diameter is less than 3 mm from the margin of the tumor.

Group C: Retinoblastoma with seeding, which can be subretinal within 3 mm of the primary tumor, vitreous seeding located < 3 mm from the primary tumor, or both vitreous and subretinal seeding < 3 mm from the primary tumor.

Group D: Retinoblastoma with diffuse seeding that may be subretinal > 3 mm from the retinoblastoma, vitreous seeding >3 mm from the retinoblastoma, or a combination of both.

Group E: Extensive retinoblastoma, which occupies more than 50% of the eye socket. It can be accompanied by neovascular glaucoma, phthisis bulbi, and/or opaque media due to hemorrhage from the anterior chamber, the vitreous or the subretinal space. Tumors with post-laminar invasion of the optic nerve, choroid, sclera, orbit, or anterior chamber also enter this section.

Trilateral and quadrilateral retinoblastoma

Trilateral retinoblastoma refers to the association of bilateral retinoblastoma with an asynchronous intracranial tumor, which occurs in fewer than 10% of bilateral cases. These tumors often arise in the pineal gland and are Primitive Neuro-Ectodermal Tumors (PNET) (pineoblastomas), but in 20-25% of cases the tumors are supra- or para-sellar (12). Rare cases of quadrilateral retinoblastoma have been reported, in which bilateral retinoblastoma is associated with both pineal-region and suprasellar intracranial primary PNET (12).

Metastatic disease in retinoblastoma

Metastatic disease occurs in 10-15% of patients and usually in association with distinct intraocular histologic features such as deep choroidal and scleral invasion, or with involvement of ciliary body, or optic nerve beyond the lamina cribrosa (12). The key to staging patients at high risk of micrometastases will probably be based in the future on circulating biomarkers in blood. One possibility is the detection of microRNAs (2).

Treatment of retinoblastoma

Rb, if untreated, can lead to death within 1–2 years (10) but, with adequate treatment, survival is better than 95% in developed countries (4, 13). Management of a child with retinoblastoma involves a balance of the patient's life with globe salvage and ultimate visual potential (11).

Enucleation (with long section, 10–15 mm, of the optic nerve) alone is curative for 85–90% of children with unilateral Rb (nonheritable) and no extraocular disease (1, 11).

Conservative treatments for Rb include intravenous chemotherapy, Transpupillary ThermoTherapy (TTT), Laser Photocoagulation (LP), CryoTherapy (CT), plaque brachytherapy (ruthenium), external-beam radiotherapy (used

in cases of progressive or recurrent intraocular retinoblastoma and extraocular retinoblastoma) and local chemotherapy delivered by subconjunctival (Carboplatin), subtenon (Carboplatin), intravitreal (Melphalan and Topotecan), or intra-arterial (Melphalan alone or combined with Topotecan) routes.

Intravenous chemotherapy is indicated in patients with bilateral (heritable) Rb, extraocular disease, intraocular disease with high-risk histologic features after enucleation, and intraocular disease in conjunction with aggressive focal therapies for ocular preservation (11).

Anti-retinoblastoma drugs include platinum compounds (carboplatin), etoposide, cyclophosphamide, doxorubicin, vincristine, and ifosfamide (11). Vincristine, carboplatin, and etoposide comprise the most frequently used combination (4).

Carboplatin is the basis of the intravenous chemotherapeutic scheme because the high levels attained in cerebrospinal fluid and vitreous humor (4).

High-risk retinoblastoma leads to metastasis in 24% of patients if not treated with systemic chemotherapy compared with 4% of those who receive it (11).

Management of retinoblastoma is a practiced art that involves tumor recognition, decision-making regarding the appropriate therapeutic approach, and meticulous follow-up for detection of tumor recurrence (11).

Second malignancies after retinoblastoma

Survivors of hereditary Rb have an increased risk for developing a subsequent malignant neoplasm, for example sarcoma or melanoma (14). Treatment with External-Beam RadioTherapy (EBRT) further amplifies this risk which is heavily dependent on the age of EBRT administration (11, 15). This risk may be acceptably small for patients older than 12 months. The cumulative risk is roughly 1% per year, reaching 50% at 50 years (16). Irradiated patients have an increased risk of soft tissue sarcomas, especially leiomyosarcomas (17). Osteosarcoma is the most common tumor outside of the irradiated field (1).

TABLE 1 Differential diagnosis of Retinoblastoma.

Coats disease
Persistent Hyperplastic Primary Vitreous
Cataract
Vitreous hemorrhage
Ocular toxocariasis
Retinal detachment
Intraocular inflammation
Meduloepithelioma
Norrie disease

Differential diagnosis of retinoblastoma

The differential diagnoses of Rb include Coats disease, Persistent Hyperplastic Primary Vitreous, cataract, vitreous hemorrhage, ocular toxocariasis and retinal detachment (2, 18). Other possible differential diagnoses comprise intraocular inflammation, retinal detachment secondary to retinopathy of prematurity, X-linked retinoschisis, meduloepithelioma, and Norrie disease (4) (Table 1).

New treatment and diagnostic perspectives of retinoblastoma

Rb is a challenging disease. Chemotherapy has been shown to have limitations during clinical practice, mainly because of the ability of Rb to become resistant to the treatment (19). So, alternative options should be available because generation of drug resistance is a factor that contributes to the failure of chemotherapy (20).

Matrix MetaloProteinase (MMP)-2 and MMP-9 possess activity against Rb at several checkpoints that are deregulated in cancer and therefore could be adjuvant therapy in patients with Rb (21).

Promising compounds for the management of Rb have been identified in preliminary phases of drug development including inhibitors of survivin, antiapoptotic Bcl-2 family proteins, methyltransferase, and kinesin proteins (22).

New treatment modalities, namely, targeted therapies, immunotherapy, and oncolytic viruses are emerging as possible non-chemotherapeutic options in Rb (23).

Pentoxifylline is a xanthine and a non-specific phosphodiesterase (PDE) inhibitor that inhibits the phosphorylation of I kappa B-alpha (IκBα) in serines 32 and 36, and this disrupts NF-κB activity. Pentoxifylline in combination with different antitumoral drugs increases the levels of apoptosis *in vivo* and *in vitro* studies and can induce increasing apoptosis in children with acute lymphoblastic leukemia (24–34). Pentoxifylline with carboplatin combination exhibited a high rate of apoptosis in human Y79 retinoblastoma cells. These findings suggest that the combination of pentoxifylline with carboplatin may comprise a promising strategy for the treatment of Rb (35).

Epigenetics is widely recognized to play a fundamental role in ocular pathologies (36). Rb tumorigenesis and progression require additional genetic and epigenetic alterations following *RB1* inactivation (37). Epigenetic dysregulation in Rb has been observed for nearly all areas of epigenetics including DNA

methylation, histone modifications, and noncoding RNAs as exemplified by promoter hypermethylation of tumor suppressor genes, activating histone modifications at the promoter of cancer pathway genes such as SYK, and aberrant regulation of microRNAs (miRNA´s) (37), and Circular RNAs (circRNAs) (38).

Long noncoding RNAs (lncRNAs) are defined as RNA transcripts longer than 200 nucleotides that have no protein-coding ability (39). LncRNA-UCA1 could promote cell proliferation and cell cycle progression and inhibit cell apoptosis in Rb by activating the PI3K/Akt pathway (39). LncRNA TUG1 has also been recognized as an oncogene in several cancers (40). TUG1 was upregulated in Rb cells and the absence of TUG1 repressed cell proliferation whereas it accelerated cell apoptosis in Rb. In brief, TUG1 is an oncogenic gene in Rb (40).

MicroRNA (miRNA) is one class of small non-coding RNA (sncRNA) that participates in a variety of biological process *via* the targeting sequence of cellular and molecular pathways (41). The oncogenic microRNA miR-17-92 has been implicated in Rb tumorigenesis (42). Expression of miR-17-92 induces rapid proliferation and disease onset. This increase in proliferation is linked to the miR-17 sub-family, which targets cell-cycle inhibitors p21 and p57 (43). miR-204 acts as a tumor suppressor, while it has much less expression in patients with retinoblastoma (44). Cyclin-D2 and MMP-9 are two key genes that are regulated by miR-204 in retinoblastoma. High expression of cyclin-D2 and MMP-9 increases the cell division rate and progression of RB (44). miR-17-3P, miR-17-5P, miR-18a, and miR-20a are highly expressed in the serum of children with Rb (45). Cone-rod homebox (CRX) and Otx-like homebox transcriptor for photoreceptor transcription have been reported as potential biomarkers in Rb (2). CTX messenger RNA is also a promising marker for the detection of micrometastases (2). miRNAs could be used as reliable biomarker for the diagnosis of RB or will be able to predict the risk of micrometastases, in the fairly near future (2, 45).

Circular RNAs (circRNAs) have vital roles in human cancers, including retinoblastoma (RB) (38). Circ-FAM158A knockdown inhibits retinoblastoma cell proliferation, metastasis and promotes apoptosis *in vitro* and *in vivo* (38). Circ_0075804 promotes RB progression through miR-138-5p-dependent regulation of PEG10 (46). CircMKLN1 overexpression slows RB progression through miR-425-5p spongylation and PDCD4 upregulation (47) and silencing circ-E2F3 inhibits proliferation, migration and invasion, and induces apoptosis of retinoblastoma cells *in vitro*, as well as reduces retinoblastoma growth *in vivo* (48). These findings could represent potential effective targets for the treatment of retinoblastoma.

Copy number alterations (CNA) have been identified and translated to current clinical practice for retinoblastoma (49). CAN reported for intraocular retinoblastoma are 1q, 2p, 6p, and 17q gains and 16q, 11q, 19q and 21q losses (49). A small percentage of patients present recurrent somatic mutations in BCL6 Corepressor gene (BCOR) (49). Analysis by Aschero et al. of CNA and BCOR gene alterations show that CNA previously reported for intraocular retinoblastoma were also found in extraocular retinoblastoma: gains in 1q, 2p, 6p, 17q and losses in 16q, 19q and 11q, in addition to BCOR alterations (49). In metastatic retinoblastoma cases included analysis of genes associated to gains in 1q (including MDM4, KIF14 genes), 2p (MYCN), and 6p (DEK, E2F3) and 16q (CDH11) deletion (49). The ATM tumor suppressor gene was significantly altered in cases with 11q deletion (49).

Clear corneal paracentesis is part of the standard intravitreal chemotherapy injection protocol (50). Kim et al. propose the extraction of the aqueous humor (AH) to be used as a liquid biopsy, or surrogate to tumor biopsy, for retinoblastoma. The safety method described by Kim et al. establishes the needles can only enter the anterior chamber and should not make contact with the iris or lens. It is most important that the needle never enters the vitreous cavity (unless combined with chemotherapy delivery), or contacts the tumor as this hypothetically elevates the risk of tumor seeding and extraocular extension of disease (50). Although clinical validity of the AH liquid biopsy platform for RB has been established, it is currently approved for research only; the AH liquid biopsy has the potential to enable precision oncology in the future, for RB (50).

Cheng and collaborators found the concentrations of IL-6, IL-7, IL-8, IFN-γ, PIGF-1, VEGF-A, β-NGF, HGF, EGF, and FGF-2 were significantly higher in the Aqueous Humor (AH) of patients with Rb than in those in the control group. These findings could contribute to the implementation of novel strategies for the diagnosis and therapy of Rb (51).

For children diagnosed with Rb, the dysregulation of methylation in *MSH6, CD44, PAX5, GATA5, TP53, VHL, GSTP1, MGMT, RB1*, and *CDKN2* genes is a further tool for targeted treatment to improve the prognosis for this ocular cancer (52).

Discussion

Patients with Rb have survival rates higher than 95-98% in industrialized countries but mortality remains high in developing countries (2, 4, 12). For example, the mortality rate in Africa is 70% (44).

In all cases of intraocular and extraocular retinoblastoma, there is a need for new therapies that are more effective and carry less risk of toxicity (23).

Epigenetic studies have shown that changes in the epigenome contribute to the rapid progression of retinoblastoma following classic genetic changes. The

targetable nature of epigenetic modifications provides a unique opportunity to optimize treatment paradigms and establish new therapeutic options for retinoblastoma with these aberrant epigenetic modifications (53).

The identification of the biomarkers and chromosomal copy number alterations described in this article could help guide future clinical management of Rb patients, for example could correlate with a more aggressive tumor (49).

Not only does the AH liquid biopsy provide the opportunity to better understand intratumoral dynamics in eyes that are actively undergoing therapy, but it also has the potential to improve patient care in the future (50).

The use of cancer screening modalities has been suggested for the goal of minimizing morbidity and mortality in the pediatric population. For example, the Bruckner test is a practical and easy test for the detection of Rb, this test consists of assessing the fundus reflex through the pupil (red reflex) in both eyes simultaneously with a bright coaxial light produced with the direct ophthalmoscope (54–65). Retinoblastoma can be detected by the Bruckner test showing a pupil that shines white or "Leukocoria". In early stages, the Rb can be detected through a minimal alteration in the Bruckner test. Early and timely diagnosis of Rb can be life-saving.

All physicians (General Practioners or Family physicians, Pediatricians, Neonatologists, Ophthalmologists, and Pediatric Ophthalmologists) who have contact with children should perform the Brucker test in order to achieve a timely detection of Rb.

Conclusion

Retinoblastoma, the most frequent malignant intraocular tumor in childhood, is caused by mutations in the retinoblastoma gene (*RB1*). Although the diagnosis of Rb remains essentially clinical, the newly identified biomarkers could contribute to early molecular detection, timely detection of micrometastases and establish new therapeutic options for Rb.

Author contributions

VMV-C, VB-C, and CCC-G contributed to conception and design of the study. CCC-G and MEC-M organized the database. VB-C, MEC-M, and CCC-G wrote the first draft of the manuscript. JCO-F, VMV-C, VB-C, and CCC-G wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Conflict of interest

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

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frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 02 November 2022
DOI 10.3389/fonc.2022.992584

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 12 July 2022
ACCEPTED 12 October 2022
PUBLISHED 02 November 2022

CITATION
Otth M and Scheinemann K (2022) Back to school – The teachers' worries and needs having a childhood cancer patient or survivor in their class.
Front. Oncol. 12:992584.
doi: 10.3389/fonc.2022.992584

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Back to school – The teachers' worries and needs having a childhood cancer patient or survivor in their class

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Background: A cancer diagnosis during childhood or adolescence causes nursery and school absences to various degrees. Attending school and meeting classmates gives many children and adolescents some normality back. Nevertheless, it can cause fears and concerns among the teachers. We are currently lacking information about the fears and needs of teachers having a child or adolescent diagnosed with cancer or with a cancer history in their classes. With this study, we aim to close this knowledge gap and assess the teachers' fears, worries and information needs having a child or adolescent diagnosed with cancer in the class to develop a suitable information tool (flyer).

Methods: We performed an online survey including teachers covering all grades from nursery to vocational school within the catchment area of our hospital. The survey included separate questions for experience with students still receiving active treatment and those in follow-up care. Answer options included tick boxes and open-ended questions, which we grouped thematically. We used descriptive analysis to describe the survey findings, resulting in a newly developed flyer.

Results: In total 358 teachers participated in the survey, 80% were female, 63% worked in nursery or primary school. One quarter (26%) had experience with a student diagnosed with cancer. Most teachers with (81%) and without (85%) experience reported at least one concern. The top three concerns reported were: (1) how to inform the class, (2) the resilience of the student and (3) how to deal with the student and his or her family. The teachers preferred oral information by physicians or parents and written information equally. Information on resilience, guidelines with an emergency situation, and the need for cancer-specific information were considered important by about 75-94% of the teachers.

Conclusion: Most teachers reported concerns, which we cover in a newly developed information flyer. However, such a flyer cannot replace individual

communication between health care professionals and teachers. The identified concerns are likely to be transferable to other school systems and countries.

KEYWORDS

pediatric oncology, school, teacher, women in science, reintegration, quality of life

Introduction

Education is an important cornerstone in a person's life. Educational achievements determine to a significant extent the future professional life. It further influences a persons' self-confidence, independence and the position in society (1, 2). A cancer diagnosis in children and adolescents often has a negative impact on school attendance and performance and may also alter or disrupt the relationship with peers and friends (3, 4). Diagnostic procedures, treatments and clinical visits often result in recurrent school absences of various durations. In addition, in treatment phases with a heavily compromised immune system school attendance is often not permitted by the treating physicians. Despite these necessary restrictions of regular school attendance, the goal is, that children and adolescents can participate at school as normally and frequent as possible. As shown by Tsimicalis et al, returning to school was perceived as very positive by the children and adolescents, gave some normality back and allowed to reconnect with peers (3). However, returning to school can be challenging for intellectual and social reasons (5, 6). Therefore, different school reintegration programs and support systems have been evaluated (7–9). For a successful school reintegration, teachers play a crucial role. Therefore, they need to be well informed and feel comfortable having an affected child or adolescent in their class. Literature and information on the potential fears, needs and uncertainties of teachers dealing with such a situation is currently missing. With this study, we aim to close this knowledge gap by describing the teachers' fears, needs and uncertainties and by providing information material based on our findings. The final goal is to make reintegration of childhood cancer patients and survivors easier by taking the teachers' needs into account.

Methods

We performed a cross-sectional, questionnaire-based survey including teachers from nursery to vocational school, working in the catchment area of our pediatric oncology center (canton of Aargau and parts of canton of Solothurn, population around 800'000). We approached the principals from elementary schools in the canton of Aargau through the cantonal authority (Kantonaler Lehrerverband), searched the remaining schools on public websites and approached their principals directly. The principals were responsible to distribute the information letter and the link to our survey to all teachers of their school.

We developed the online survey for the purpose of this study (Supplemental Explanation E1). Answer options included tick boxes and open-ended questions, which we grouped thematically. The survey differentiated between teachers' concerns, fears and information needs having a student newly diagnosed with cancer or a cancer survivor who completed treatment already. The survey had further separate sections for teachers with and without experience of having a childhood cancer patient or survivor in their class. We performed a pilot phase of the survey with three teachers from different school levels. They gave feedback on the surveys' comprehensibility and structure. Following the implementation of their feedbacks, we distributed the information letter and the survey link, using SurveyMonkey®. We mainly performed descriptive analysis and used t-test and chi squared test to compare characteristics of different groups of teachers. For these analyses, we used STATA 17.0 and p-values <0.05 were considered statistically significant.

Results

We approached 338 schools or principals and received feedback from 417 teachers. We excluded 59 teachers, who only completed the part on personal characteristics, but skipped all questions on fears and concerns. The characteristics of these teachers did not differ from those who completed the whole survey (Supplemental Table S1). Most teachers were female (80%) and worked in primary school levels (63%). Half of the teachers (51%) were the principal teachers of a class. The median time of working experience was 18 years (Interquartile Range, IQR 8 – 25) (Table 1).

One quarter of the teachers (n=94; 26%) reported having experience with a child or adolescent newly diagnosed with cancer or a childhood cancer survivor. Of those, 43 teachers reported that they received specific information. They received the information mainly from the parents (58%) or a

TABLE 1 Characteristics of participating teachers (n=358).

	Number (%)
Sex	288 (80)
Female	
Age	
18 – 34 years	83 (23)
35 – 44 years	81 (23)
45 – 54 years	100 (28)
55 – 64 years	90 (25)
65 years or older	4 (1)
Working years [years] Median (IQR)	18 (8 – 25)
School level*	
Primary level	226 (63)
Secondary level I	100 (28)
Secondary level II	147 (40)
Role	
Principal teacher of a class	181 (51)
Individual school subjects	119 (33)
Other	58 (16)
Region	
Rural	192 (54)
Urban	166 (46)
Experience	
Yes	94 (26)
No	264 (74)
Brochure helpful	
Yes	264 (74)
No	77 (21)
Missing	17 (5)

*more than one level possible per teacher; explanation in Supplementary Explanation E1.

combination of parents and health care professionals (19%) (Table 2). The main topics were about the students' resilience (84%), the specific type of cancer (70%) and possible emergency situations for the child (35%). The information was sufficient for most teachers (72%) and was mainly delivered orally by parents (79%) or health care professionals, including physicians or hospital teachers (21%) (Table 2).

Further 315 teachers did not have a child or adolescent diagnosed with cancer in their class or they had one in their class, but did not receive information. These teachers would prefer receiving information from parents (71%), written information material (65%) or orally by health care professionals (60%) (Table 2). As for teachers with experience with childhood cancer patients or survivors, the three most relevant topics would be on students' resilience (95%), possible emergencies (87%) and the specific type of cancer (75%) (Table 2).

The fears and concerns mentioned by the teachers resulted in seven thematical groups: 1) students' resilience; 2) dealing with the student and his or her family; 3) dealing with the topic of relapse and death; 4) possible emergencies; 5) infections and hygienic measures at school; 6) informing and dealing with classmates; 7) teachers' own feelings (Table 3). No teacher reported any concerns related to potential legal responsibilities.

Most teachers with (81%) and without (85%) experience reported at least one fear or concern (Figure 1C). How to inform the classmates (41% and 51%) and the students' resilience (38% each) were the most frequently reported concerns in both groups. The topic of death and relapse was reported by a larger proportion of teachers with experience (29%) compared to those without (23%) experience. The topic on how to deal with the student and his or her family was raised more frequent by teachers without experience (34%) compared to those with experience (27%).

Looking at the teachers' feedbacks having a student under active treatment in their class, 77% of teachers with experience reported at least one concern and 78% of teachers without experience (Figure 1A). The three most frequently mentioned concerns in teachers with experience were students' resilience (31%), how to deal with classmates (30%) and the teachers' personal concerns (19%). Teachers without experience reported most frequently concerns about classmates (39%), students' resilience (28%) and how to deal with the student or his or her family (19%).

Looking at the teachers' feedbacks having a student in the class who completed the treatment already, the distribution of each of the concerns is similar to teachers who have a student under active treatment, but the proportions of each concern are lower (Figure 1B). Only 39% of teachers with experience report any concern and 50% of teachers without experience. Again, students' resilience and the topic of death and relapse were reported most frequently.

Most teachers consider it important to inform the classmates having a child or adolescent with cancer (85%, Table 4). Most teachers prefer to be involved informing the classmates (76%), 33% prefer the involvement of physicians and one fourth (26%) a written document (Table 4). More than 80% of teachers prefer to inform the classmates about what the treatment means for the affected child or adolescent and how they can participate in school lessons and sports. Half of the teachers (Table 4) prefer information about cancer in general (e.g. the meaning of the word "cancer", different types of cancer and cancer treatment).

The topics mentioned in this survey resulted in the development of a short flyer. The flyer covers the following topics (one page each): 1) childhood cancer in Switzerland in general, 2) a child or adolescent receiving active treatment, 3) a child or adolescent, who already completed treatment, 4) dealing in everyday school life, 5) contact details and further literature and information (websites and books about the topic).

Discussion

Our results show, that over 80% of teachers reported fears, concerns and uncertainties, independent of whether they had experience with a student diagnosed with cancer or not. The

TABLE 2 Information received or needed stratified by teachers' experience with children diagnosed with cancer.

	Number (%)
Teachers who had a child diagnosed with cancer in their class and received information (n = 43)	
What was the situation of the child?	
New diagnosis	34 (79)
Treatment finished	8 (19)
Missing	1 (2)
What was the source of information?	
Hospital	2 (5)
Parents	25 (58)
Hospital and parents	8 (19)
Other	7 (16)
Student him-/herself	4
Another teacher	2
Myself	1
Missing	1 (2)
What type of information did you receive?	
Brochure from hospital	5 (12)
Oral by physician, incl. hospital visit, hospital school	9 (21)
Parents	34 (79)
Link to homepage	0
Other	6 (14)
Student him-/herself	2
Another teacher	4
Which information did you receive?	
Cancer in general	10 (23)
Cancer specific	30 (70)
Who to call in case of an emergency	18 (42)
Possible emergency situations	15 (35)
Student resilience	36 (84)
Was the format of information appropriate?	
Yes	35 (81)
No*	4 (9)
Missing	2 (5)
Was the information sufficient?	
Yes	31 (72)
No°	7 (16)
Missing	2 (5)
Teachers who did not have a child diagnosed with cancer in their class or had a child in their class bur did not receive information (n=315)	
What would be the preferred source of information?	
(multiple answer options)	205 (65)
Flyer	188 (60)
Physicians	224 (71)
Parents	51 (16)
Other‡	
Which information would be important?	
(multiple answer options)	142 (45)
Cancer general	235 (75)
Cancer specific	108 (34)
Who to call in case of an emergency	275 (87)
Possible emergency situations	297 (94)
Student resilience	54 (17)
Other (all covered in the other survey sections)	

* written information would have been better (n=3); only information from student (n=1).
° missing background knowledge (n=1); regular updates (n=2); contact point for questions (n=3).
‡ Child/adolescent and/or parents (n=19); school principal (n=8); specific contact point (n=5); hospital school, information event, webpage, round table (n=3 each); by parents and physicians together, childhood cancer Switzerland (n=2 each); hospital visit, video, children's book (n=1 each).

topics reported most frequently did not differ between the situation of a child or adolescent newly diagnosed with cancer compared to a cancer survivor. Our results underline, that there is an information need among most teachers how to handle these new situations appropriately.

The concerns reported by teachers from our study are comparable to the information needs of teachers reported by Brown et al. (10) and Chekryn et al. (11). Brown et al. assessed the needs of 528 teachers to develop a computer-based training program. One section asked the teachers to state areas where additional information would be helpful. Physical limitations (89%), talking to the child (84%) or the child's classmates (84%), and the emotional impact on the families (76%) were reported most frequently (10). These areas were also considered important by teachers from our survey. Chekryn et al. interviewed nine children, parents and teachers 4-6 weeks following the return to school. Uncertainties were related to academic expectations, information needs on the disease and its course versus the patients right for privacy, how to talk about emotions, and the personal impact on the teachers, including the feeling of being unprepared (11). Even though Chekryn et al. published their results in 1987, the topics are still comparable to those mentioned in our survey. This is supported by the results from Fryer et al. published in 1989 (12). Their survey was answered by 33 teachers, who reported becoming too emotional, especially explaining to the class a student's death, as the main concerns. The publication by Chekryn et al. also highlighted the important aspect of privacy (11). Nowadays, it is considered obvious, that any information about the child or adolescent can only be given with his or her consent or the parent's consent. This privacy must be respected and is defined differently and individually by each child, adolescent or their parents.

In 2002, Papadatou et al. performed a survey to explore the experiences of Greek teachers regarding the school integration of children and adolescents diagnosed with a chronic or life-limiting condition (13). From the 1792 respondents, 19% (n=340) reported having experience with a chronically sick child. Cancer was the second most frequent condition reported by the teachers with experience. Asked about situations, that affected them most, they reported most frequently 1) the inability to handle emergencies, 2) the child's physical changes and 3) the child's strategies to cope with the illness (13). Participants from our survey also emphasized the first two topics. Two third (60%) of Greek teachers recognized changes on communication and behavior towards the sick child. They avoided pressure and criticism, had fewer expectations for academic performance and showed greater support. Teachers, who did not report changes in their attitudes, tried not to differentiate between the sick child and the classmates. This dichotomy was also reported by the participants form our survey. The perception of the Greek teachers might not directly be comparable to our cohort as childhood cancer treatment is often an intense phase of a given duration, often followed by a slowly returning normality. This is often not the case for other

TABLE 3 Fears and concerns mentioned by the teachers, sorted in seven thematical groups (examples only).

Students' resilience	«what can I ask from the child?»; «not to under- or over-challenge the child»; «physical and mental resilience»
Dealing with the student and its family	«respect for communication with the child»; «how can I help properly»; «how to deal with the child in everyday life»
Dealing with the topics of relapse and death	«dealing with the topic of death»; «I would have to think carefully how to integrate the topic of health, life, future perspectives and death in the lessons»
Possible emergencies	«how do I react correctly in case of an emergency»
Infections and hygienic measures at school	«risk of infectious diseases that could endanger the child»; «information on hygienic measures»
Informing and dealing with classmates	«how and what to communicate to the class»; «reaction of classmates»
Teacher	«how do I deal correctly with such a situation as a teacher»; «fear to find the correct words»; «fear of the unknown»; «respect of not behaving correctly»

chronic diseases, such as sickle cell anemia, epilepsy, paralysis or diabetes, which persist life-long. These differences in anticipated duration of the disease time might influence the teachers' attitudes towards the sick child, but also the current situation of the health status and the prognosis. These factors again influence the teachers' potential fears and uncertainties.

Different school reintegration programs are described today, and teachers and school staff are involved to different degrees (8). Thompson et al. conclude that these programs are very much appreciated by patients, parents and school staff. They contribute to a better understanding among teachers and classmates regarding the disease and its consequences for the sick child (8). School reintegration programs might be the right place to provide information about the topics raised in our survey.

Annett et al. performed a feasibility study and evaluated a school reintegration intervention for children diagnosed with acute lymphoblastic leukemia. The intervention lasted over 4 months and was performed by a family advocate (7). The intervention consisted of eight consecutive modules: 1) getting to know you and your child, 2) communication between family, school and hospital, 3) communication with school personnel, 4) treatment effects upon the sibling(s), 5) advocacy, 6) short-term effects of acute lymphoblastic leukemia treatment 7) long-term effects of acute lymphoblastic leukemia treatment, 8) closure/review. The authors conclude, that the intervention proved to be satisfactory in the eight participating families (7). However, all families were off treatment already, and the perspective of teachers' fears and concerns seems not to be part of any module. From a teachers' perspective and based on our results, we advocate on their early involvement. A first module could for instance achieve this at the time of cancer diagnosis. In addition, addressing potential fears and concerns proactively by the health care providers can give the teachers a trustful relationship and influence the future collaboration positively. This also gives the teachers an opportunity to address topics, that they may not dare to address spontaneously, such as being afraid to be too emotional or to be unsure on how to deal with the child and his/her parents when they meet the first time following the diagnosis. Several publications highlighted that information is a crucial element to reduce the fears and uncertainties of teachers having a child with cancer or another long-term health condition in their class (10, 14, 15). In 1992, Katz et al. evaluated a school reintegration intervention, conducted by hospital-based pediatric psychologists (16). The intervention had the following five components: 1) preventive education including the information of teachers and school personnel by phone, 2) identification of a hospital-based liaison, 3) preparation of the child's return to school, 4) informative presentations to school personnel and classmates, 5) periodic follow-up. The fourth component is targeted to the teachers' needs and to inform them adequately. This might be the right place again to proactively address the fears and concerns mentioned in our survey.

In 1988, Stevens et al. assessed, how hospitals in the United Kingdom liaised with the schools of a child diagnosed with cancer (17). They explicitly asked about the provision of written information. Five out of 13 participating hospitals used written documents specifically designed for the teachers. The documents combined covered the following five topics: overview of childhood cancer, risk of infection and medical problems at school, children's knowledge of their disease, school attendance and academic performance (17). These topics are also covered in our flyer. This highlights, that even though the field of pediatric oncology and school system changed a lot since 1988, the main information needs remained the same.

Limitations and strength

Approaching the principals from elementary schools in the canton of Aargau through the cantonal authority only and searching the remaining schools on public websites may have resulted in a participation bias. It made sending-out reminders impossible. Through this approach, we were also not able to calculate the response rate. A personalized mail to each teacher may have increased the return of completed questionnaires. Even though we tried to locate all schools in the catchment area, we

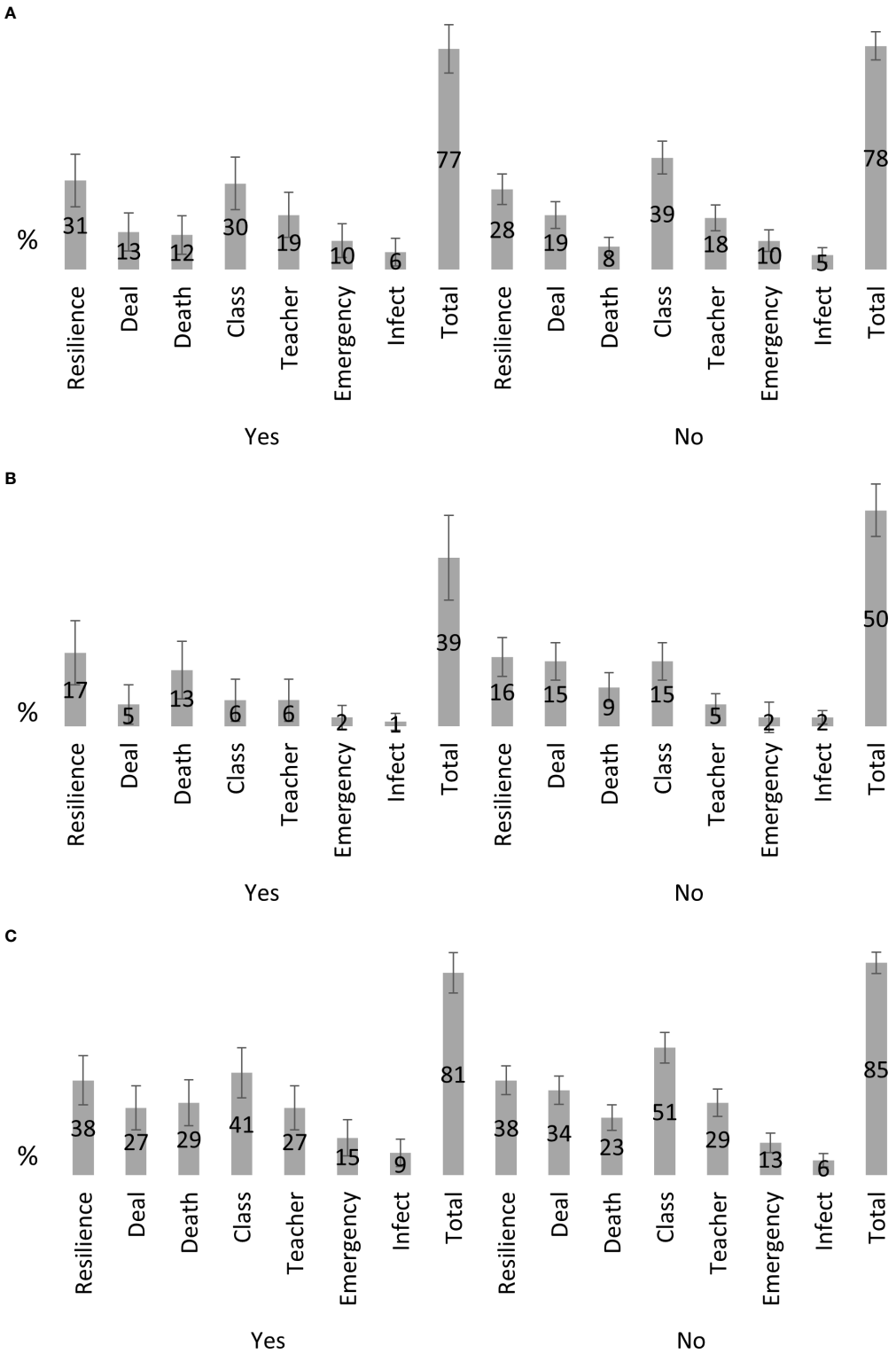

FIGURE 1
Frequency of concerns reported by teacher (n = 358); **(A)** concerns if a student receives active treatment; **(B)** concerns if a student completed treatment; **(C)** any concerns combined

TABLE 4 Teachers' perception if/how to inform classmates (n=358).

	Number
Is it important to inform the classmates?	
Yes	307 (86)
No	37 (10)
Missing	14 (4)
How should classmates be informed?	
Written information/document	94 (26)
Physician	118 (33)
Teacher	274 (76)
Other*	155 (43)
Which information should classmates receive?	
Cancer general	191 (53)
Cancer treatment	194 (54)
What treatment means for the affected child	320 (89)
Participation school, sports	304 (85)
Death	172 (48)
Other	55 (15)

*most statements include individual approach and depending on the patients/parents preferences; child/adolescent himself if possible [n=43]; parents [n=17]; parents and/or child/adolescent [n=21]; social worker or psychologist from school [n=7]; video or books [n=4]; hospital tour [n=2].

might have missed some. Questionnaire-based studies have the inevitably risk of participation bias, resulting in a selected population. It might be that, more anxious teachers participated in the survey or those, who already had a childhood cancer patient in their class, but did not receive enough information. We did not collect detailed information from teachers, who received information to assess whether the needs changed over time. It was statistically not possible to compare teachers, who received information versus those without as the difference in the number of teachers was too big. Despite these limitations, the strengths of this study are the participation of teachers from all different school levels and the relatively large sample size. Therefore, we think that the results are generalizable for all teachers in Switzerland and even for other countries with similar healthcare and educational systems. Evaluating the impact of the flyer was not part of this study and has to be assessed prospectively.

Conclusion

Teachers do have specific needs having a childhood cancer patient or survivor in their class. Knowledge of these needs is crucial to address them appropriately and to facilitate school reintegration of the affected child or adolescent. A flyer, such as the one developed within this study, covering topics common for all childhood cancer patients and survivors, may be a first guidance, but has to be evaluated in a future step. However, from the setup and clinical experience, such a flyer cannot replace an individual approach for each childhood cancer patient or survivor, neither the direct communication between patients, parents, health care professionals and teachers.

Data availability statement

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

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization, KS and MO. Data collection, MO. Formal analysis, MO. Funding acquisition, MO. Writing - original draft, MO. Writing - review and editing, KS and MO. All authors contributed to the article and approved the submitted version.

Funding

Research Council (Forschungsrat) Kantonsspital Aarau.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.992584/full#supplementary-material

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frontiers | Frontiers in Pediatrics

TYPE Original Research
PUBLISHED 04 November 2022
DOI 10.3389/fped.2022.950911

Check for updates

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Pediatrics

RECEIVED 23 May 2022
ACCEPTED 27 September 2022
PUBLISHED 04 November 2022

CITATION
Puglisi F, Soma R, Podda M, Vetrella S, Rabusin M, Tropia S, Meli M, Russo G, Sorrentino S, Erminio G, Pulvirenti A, Ruggieri M and Di Cataldo A (2022) Neuroblastic tumors and neurofibromatosis type 1: A retrospective multicenter study in Italy and systematic review of the literature.
Front. Pediatr. 10:950911.
doi: 10.3389/fped.2022.950911

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Neuroblastic tumors and neurofibromatosis type 1: A retrospective multicenter study in Italy and systematic review of the literature

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Background: Neuroblastic tumors (NBTs) are the most common extra-cranial solid tumors of childhood. Neurofibromatosis type 1 (NF1) is the most common neurocutaneous disorder with a predisposition to tumors. The co-occurrence of NBTs in the setting of NF1 has been occasionally reported, suggesting a non-casual association and likely configuring a spectrum of neural crest–derived disorders.

Aim of the study: To explore the occurrence of NBTs within NF1 and to report on its natural history, therapeutic strategies, and outcomes in an Italian cohort of children with NF1 and in the literature.

Subjects and Methods: Study (a): a retrospective analysis of questionnaire-based data [years 1979–2017] derived from the databases of the Italian Registry for Neuroblastoma (RINB) of the Italian Society of Pediatric Onco-Haematology (AIEOP); and Study (b): a systematic review search on NF1/NB co-occurrence.

Results: Study (a) identified eight children with NBTs, 0.2% of patients registered in the RINB, fulfilling the diagnostic criteria for NF1. The primary site of NBTs was abdominal in six patients. The NBTs were neuroblastoma (NB) in five patients, ganglioneuroblastoma (GNB) in one, patient, and ganglioneuroma (GN) in two. Metastatic diffusion occurred in three out of eight children. *MYCN* gene testing, performed in the tumors of five patients, resulted not-amplified. The major features of NF1 included the following: NF1 family history in four patients, café-au-lait spots in all, freckling in six, Lisch nodules in three, and neurofibromas in three. With regard to the outcome, four children survived three of these for the progression of NB

10.3389/fped.2022.950911

and one for a second tumor. Study (b) identified 12 patients with NF1/NB from the years 1966–2017, and the median age at diagnosis was 27 months (range = 0–168 months). The primary site of NB was thoracic. The prevalent histotype was NB in nine patients, GNB in two, and GN in one. Eight/nine NBs were metastatic. The *MYCN* gene was amplified in the only studied case. The NF1 features included NF1 family history in seven patients; the major NF1 features were café-au-lait spots in nine patients, freckling in one, Lisch nodules in none, and neurofibromas in six. The outcome was good for only two children, while eight children died of neuroblastoma, at a median age of 49.5 months (range = 2.4–174 months), with a median survival time of 21.75 months after diagnosis.

Conclusions: To our knowledge, this represents the first systematic study on the occurrence of NBTs in NF1. This confirms that NBs are rare *per se* in the setting of NF1 (0.2% of all NBs) and even if compared to the overall frequency of malignancies in NF1 (i.e., 14.7%). The male:female ratio in study (a) (0.6) was different from what was recorded in study (b) (1.5) and in line with the overall increased frequency of malignancies in females with NF1. The median ages at diagnosis of NB in either study (a) or (b) were concordant with what occurred in the NB population. In study (a) versus study (b), the frequency of metastatic diffusion was lower, likely indicating less awareness on work-ups for malignancies in old NF1 series in the literature. The outcome was much better in study (a) than in study (b), indicating that multidisciplinary treatment for NB is highly recommended.

KEYWORDS

neurofibromatosis type 1, NF1, neuroblastoma, child, cancer

Introduction

Neuroblastic tumors (NBTs) are very rare tumors, characterized by various clinical presentations and diverse prognosis of its subsets. While in some patients the tumor is successfully treated with surgery alone, or may regress spontaneously, the chances of cure in children older than 1 year with metastatic disease remain poor. Although the probability of survival for children with NBTs has improved over time, even the best published results do not parallel those obtained for most other childhood malignancies (1). A few studies have documented this finding by reporting a large series of patients diagnosed over a long period (2–6).

Most NBTs are sporadic and not correlated with any specific constitutional germline chromosomal abnormality, inherited predisposition, or associated congenital anomalies. Nevertheless, there are some exceptions. A higher incidence of NBTs has been suggested in girls with Turner syndrome (7). Patients with Kabuki syndrome and NBTs have been reported (8, 9). Hirschsprung's disease, congenital central hypoventilation (Ondine's curse), and neurofibromatosis type 1 (NF1) have all been described in association with NBTs, suggesting the existence of a global disorder of neural crest–derived cells (i.e., neurocristopathy) (10–13).

NF1 is the most common form of neurofibromatosis and one of the most common autosomal dominant disorders in humans, with an incidence of 1 in 2,600–3,000 individuals (14, 15). Approximately one half of the cases are familial (inherited). The remainder are the result of *de novo* (sporadic) mutations (16). These mutations occur primarily in paternally derived chromosomes, and the likelihood of *de novo* NF1 increases with advanced paternal age (17). The incidence of segmental NF1 is estimated at 1 in 36,000–40,000 (18).

Patients with NF1 are predisposed to both benign and malignant tumors of neurogenic and non-neurogenic origin. Most studies that have addressed the risk of malignancy and early death have shown an approximately 8- to 15-year decrease in life expectancy in patients with NF1, mainly as a result of malignancy (19, 20). Mortality in NF1 has previously been studied in cohorts from France, Wales, United States, and Denmark (21–23). These studies have shown excess mortality rates of NF1 patients compared with those of the general population and a high proportion of deaths caused by malignancies.

Multiple studies have shown a substantial risk of nervous system malignancy, with an indisputable excess risk of gliomas and malignant peripheral nerve sheath tumor (MPNST) (24), both of which result in an excess risk of mortality (25). The onset of NBTs in patients with NF1 has also been described, but the actual incidence of this association is not known (26).

The purpose of this study is to analyze the clinical–epidemiological characteristics of this rare clinical association, as well as the therapeutic approach and prognosis, in order to allow a descriptive analysis of the development of clinical and research experience.

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Materials and methods

Setting and sample

All patients with a diagnosis of a NBT and NF1 were included in this analysis. The cases were identified by searching the databases of the Italian clinical units of pediatric onco-hematology and the Italian Neuroblastoma Registry (RINB) database (from 1979 onward). The RINB was activated in 1979 and includes all subjects with any peripheral NBT [i.e., neuroblastoma (NB), ganglioneuroblastoma (GNB), and the benign ganglioneuroma (GN)], diagnosed at the institutions included in the Italian Neuroblastoma Group (ING) of the Italian Association of Pediatric Hematology and Oncology (AIEOP) (27). More than the expected number of NBT patients from Italy have been recruited through this network (28).

The data on the included patients were collected retrospectively: patients diagnosed from the years 1979 to May 2017 were considered. The patients were treated according to the AIEOP protocol till the 1990s and European SIOPEN protocols thereafter. Informed consent to the treatment and to data collection and analysis was obtained from all patients according to institutional guidelines at the time of enrolling patients in the protocols.

A standardized questionnaire was sent to all the 54 hematology-oncology units belonging to the AIEOP with the aim of collecting information about the two conditions. The survey was designed to assess data regarding epidemiological aspects, clinical features, and genotype/phenotype correlation and the management and course of the patients individuated. The questionnaire included two parts, one relating to a description of the NBT and the other relating to NF1, as given in **Tables 1**, **2**. Tumor diagnosis, disease extension, and response to treatment were defined according to the International Neuroblastoma Staging System (INSS) and the International Neuroblastoma Risk Group (INRG) staging system as described previously (29, 30). The long-term sequelae were ascertained by contacting the clinical investigators at the various centers; no additional investigations were conducted on possible late effects for the purposes of this study.

Statistical analysis was conducted within the R statistical System. To identify possible correlations among the subgroup of patients, we performed principal component analysis using the variables characterizing both NF1 and NBTs.

TABLE 1 Characteristics of the tumor in eight patients.

Patient No.	1	2	3	4	5	6	7	8
Clinical presentation								
Sex	Male	Female	Female	Male	Female	Female	Female	Male
Age at diagnosis (months)	30	68	92	57	23	24	120	24
Symptoms at diagnosis	No	Dysuria and vulva edema	No	Abdominal pain	No	Abdominal distension	Rapidly growing neurofibroma	No
Primitive tumor site	Abdomen (adrenal gland)	Pelvis	Abdomen (adrenal gland)	Abdomen (adrenal gland)	Pelvis	Abdomen (ganglia)	Abdomen (ganglia)	Abdomen (adrenal gland)
Histology	NB (NOS)	GN	NB (NOS)	NB (NOS)	GN	NB (NOS)	GNB nodular	NB (NOS)
Diagnosis procedure	Biopsy	Biopsy	Biopsy	Bone marrow aspirate	Biopsy	Biopsy	Biopsy	Biopsy
Dumbbell tumor	No	No	No	No	Unknown	No	No	No
Staging								
INSS	2A	NA	4	4	NA	4	3	1
INRG	L1	NA	M	M	NA	M	L2	L1
Metastases	No	NA	distant lymph nodes, bone	bone marrow, bone, distant lymph nodes, and orbit	NA	bone marrow	No	No
Biochemical data								
Serum LDH	Normal	Normal	Elevated	Elevated	Normal	Elevated	Normal	Elevated
Urine VMA	Unknown	Normal	Elevated	Unknown	Unknown	Elevated	Normal	Unknown
Serum Ferritin	Unknown	Normal	Unknown	Unknown	Unknown	Normal	Unknown	Unknown
Molecular data at onset								
MYCN status	Normal	Not Done	Normal	Not Done	Not Done	Normal	Normal	Normal

GN, ganglioneuroma; GNB, ganglioneuroblastoma; INRG, International Neuroblastoma Risk Group Staging System; INSS, International Neuroblastoma Staging System; LDH, lactate dehydrogenase; NA, not applicable; NB, neuroblastoma; NOS, not otherwise specified; VMA, vanillylmandelic acid.

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TABLE 2 Characteristics of NF1 in eight patients.

Patient	1	2	3	4	5	6	7	8
Sex	Male	Female	Female	Male	Female	Female	Female	Male
Age at diagnosis (months)	15	30	84	8	12	24	120	8
Pregnancy	Unknown	Unknown	Normal	Normal	Unknown	Normal	Normal	Macrosomia
NF1 family history	Unknown	Mother	No	No	Mother Brother	Father	No	Father
Perinatal period	Phototherapy for jaundice	Unknown	Normal	Normal	Patent foramen ovale	Acute pyelonephritis	Normal	Normal
NF1 features								
				Major features				
Café-au-lait spots								
Trunk	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Arms	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Legs	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Freckling								
Axillae	Yes	Yes	Yes	Unknown	Yes	Yes	Yes	No
Groin	Yes	No	Yes	Unknown	Yes	Yes	Yes	No
Neck	No	No	Yes	Unknown	Yes	Yes	No	No
Perioral	No	No	No	Unknown	No	No	No	No
Lisch nodules	Bilateral	Unknown	Bilateral	Unknown	No	No	No	Monolateral
Neurofibromas								
Trunk	No	No	No	No	Yes	No	Yes	No
Arms	No	No	No	No	Yes	No	Yes	No
Legs	Yes	No	No	No	Yes	No	Yes	No
Minor features								
Short stature	No	Yes	Yes	Unknown	No	No	No	No
Head circumference	Macrocephaly	Normal	Macrocephaly	Macrocephaly	Unknown	Macrocephaly	Normal	Macrocephaly
Dysmorphic features	No	No	Yes	Yes	Yes	No	Lumbar scoliosis	No
Complications	Unknown	No	Optic glioma	Unknown	Bilateral Hydronephrosis (with nephrostomies) Pelvic plexiform neurofibromas Clitoral hypertrophy	No	No	No
Cognitive profile	Normal	Normal	Normal	Cognitive deficit	Unknown	Unknown	Normal	Normal
Behavioral profile	Unknown	Normal	Normal	Psychomotor deficit	Unknown	Hyperactivity	Normal	Normal
Brain MRI	Hamartoma	Unknown	Optic glioma	Not done	Optic glioma UBOs	Not done	UBOs	Not done
Spinal MRI	Not done	Unknown	Normal	Not done	Neurofibromas with spinal cord invasion and compression, MPNST	Not done	Hydromyelia	Not done
NF1 gene analysis	Unknown	Not done	Not done	Unknown	Positive	Positive	Positive	Positive

MPNST, malignant peripheral nerve sheath tumor; MRI, magnetic resonance imaging; UBOs, unidentified bright objects.

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Results

Demographic information

Overall, eight patients with NBTs and NF1 were registered, who included three males and five females, with a male:female ratio of 0.6. They represented 0.2% of all patients registered in the RINB (3,976 patients). The age at diagnosis of neuroblastoma was between 23 and 120 months, with an average of 54.7 months and a median age of 43.5 months. The age at diagnosis of NF1 was between 8 and 120 months, with an average of 37.6 and a median age of 19.5 months. The patients' demographic data are given in Table 3.

Clinical presentation

The abdomen was the primary site for six patients, the adrenal gland for four patients, paravertebral ganglia for two, and pelvis for two. Symptoms at diagnosis were present in half of the patients and depended on the primary site: one child had dysuria and vulva edema, two had abdominal pain, and only one patient had a symptom correlated with NF1, an increase in the size of a neurofibroma in the same site. No patient had a dumbbell tumor (Tables 1, 2).

The histology most represented was NB in five patients. One patient had a GNB nodular, and two had GN. Among these five patients with neuroblastoma, metastatic disease was present at onset in three patients; the other two cases with NB,were both stage L1 for the INRG and stage 2A and 1 for the INSS. The sites of metastasis were distant lymph nodes, bone, and bone marrow in two patients and orbit in one.

With regard to biologic studies at diagnosis, serum lactate dehydrogenase (LDH) was studied in all patients and was found to be above normal value in four patients; serum ferritin was normal in the two studied cases, while urinary vanillylmandelic acid (VMA) was above normal value in two of the four studied cases. The *MYCN* gene was not amplified in the five studied cases.

In six patients, NF1 was diagnosed before the diagnosis of NBTs, while in two patients, it was diagnosed at the same time. Half of the patients had a family history of NF1. The perinatal period was uneventful for four patients, one was treated for jaundice at birth, one was treated for urinary tract infection, and one had a patent foramen ovale at birth. At diagnosis, café-au-lait spots were found on all patients, distributed over the trunk, arms, and groin in all of them; freckling was recorded in seven patients; bilateral ocular Lisch nodules were detected in two patients and monolateral ocular Lish nodules in one. Three children developed neurofibromas. For what was regarded as minor features, macrocephaly was reported in five patients, short stature in two, and dysmorphic features in four. With regard to the cognitive and behavioral profile, one case of hyperactivity was reported and there was one case of cognitive and psychomotor deficit. On brain magnetic resonance imaging (MRI), optic glioma was detected in two patients and hamartoma in one; unidentified bright objects (UBOs) were observed in two. Furthermore, spinal MRI showed the presence of neurofibromas with a compression of the spinal cord in one patient and hydromyelia in another. Genetic testing was performed in four patients to confirm the diagnosis. Patient No. 5 was the one who presented the most significant number of complications related to NF1, with the presence of bilateral hydronephrosis (with nephrostomies), clitoral hypertrophy, pelvic plexiform neurofibromas, neurofibromas with invasion of the spinal canal, and a compression of the spinal cord and MPNST.

Because of the availability of only a few samples, we were not able to establish any statistically significant relationship among these patients.

TABLE 3 Demographic information of the study patients.

Characteristic	All patients ($n = 8$)
Sex, No.	
Male	3
Female	5
Age in months at diagnosis of NB	
Mean	54.7
Median	43.5
Range	23–120
Age in months at diagnosis of NF1 (evaluated only in six patients)	
Mean	37.6
Median	19.5
Range	8–120

Treatment

Treatment and outcome are summarized in Table 4. As first-line treatment, surgery was performed on two patients, and it involved a complete resection. No postoperative complications were reported. Four patients received multidrug chemotherapy according to their time protocols. Only one patient received, after induction chemotherapy, a consolidation treatment with megatherapy and stem cell rescue, radiotherapy, immunotherapy, and cis-retinoic acid. Only one patient underwent 131I-metaiodobenzylguanidine (MIBG) therapy, given the intense capture of the documented mass with MIBG scintigraphy.

Outcome

Three patients reached the stage of complete remission (CR) after first-line treatment, two reached the surgery alone stage,

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TABLE 4 Treatment details and outcome for eight patients.

Patient no	1	2	3	4	5	6	7	8
Treatment								
Surgery first line	Complete resection	No	No	No	No	No	No	Complete resection
Chemotherapy first line	No	No	Yes	Yes	No	Yes	Yes	No
Megatherapy with stem cell rescue	No	No	Yes	No	No	No	No	No
Radiotherapy first line	No	No	Yes	No	No	No	No	No
Therapeutic MIBG	No	No	No	No	Yes	No	No	No
Immunotherapy	No	No	Yes	No	No	No	No	No
Cis-retinoic acid	No	No	Yes	No	No	No	No	No
Response to treatment and outcome								
Response	CR	SD	DP	DP	DP	DP	CR	CR
Relapse/Disease Progression	No	No	DP After first-line therapy	DP During first-line therapy	DP after MIBG therapy	DP After first-line therapy	No	No
Rescue treatment	No	No	Chemotherapy	Chemotherapy	Chemotherapy	Chemotherapy	No	No
Outcome (Months after diagnosis)	Alive CR (44,5)	Alive SD (96)	Death from DP (19)	Death from DP (6)	Death caused by MPNST (unknown)	Death from DP (20)	Alive CR (7)	Alive CR (53)

CR, complete response; DP, disease progression; MIBG, 131I-metaiodobenzylguanidine; MPNST, malignant peripheral nerve sheath tumor; SD, stable disease.

and one the chemotherapy stage, and these patients are in CR at 44.5, 53, and 7 months, respectively, after diagnosis; only one patient was observed and is in a condition of stable disease (SD) 8 years after diagnosis. Disease progressed in four patients after first-line therapy in two, during first-line therapy in one, and after MIBG therapy in one. All of them received rescue chemotherapy, but they all died, three of NB at 6, 19, and 20 months from diagnosis and one from a second tumor (mediastinal MPNST), but their time of death was not reported.

Literature review and discussion

A literature study was performed using MEDLINE/PubMed up to May 2017. The following MeSH headings and text words were used: "neuroblastoma AND neurofibromatosis type 1" and "neuroblastoma AND von Recklinghausen disease". We did not impose any language restrictions. Two additional articles from the reference lists were obtained. Finally, all papers identified in the literature scan were examined and relevant papers were reviewed and summarized for inclusion in this report (31–38) (**Figure 1**).

Overall, 12 cases of patients with NBTs and NF1 were reported between 1966 and 2013: 7 males and 3 females (the sex of the other two patients was not available). The age at diagnosis of NBTs was between 0 and 168 months, with an average of 39 months and a median age of 27 months. The age at diagnosis of NF1 was specified only in five patients, while in three patients, it was not reported, and in four patients, NF1 was diagnosed at the same time of NB diagnosis. The patients' demographic data are presented in **Table 5**.

A detailed description of the case reports is given in **Tables 6A,B**. The primary site of NBTs was known in 11 out of 12 patients. It was in the mediastinum in five patients, in the abdomen in four patients, and in the neck in one, and in one patient, there were two masses, both in the thorax and in the abdomen. Diagnostic symptoms were present in all patients and correlated with the primary site. Dumbbell tumor was found in two patients, one with NB and one with GNB.

The histotype was NB in nine patients, GNB in two, and GN in one. One diagnosis of NB (patient 12) was done on liver biopsy, while the primary thoracic tumor, whose histology was analyzed some months later, was a ganglioneuroma, probably due to the maturation of a NB. Among these nine patients with NB, eight were metastatic. In one patient with GNB, metastasis appeared at relapse in the bone and bone marrow.

The results of the biologic studies were reported only for six patients. Above normal values were recorded in two patients for LDH and in three for VMA. An *MYCN* gene study was performed in only one patient and it was amplified.

Seven patients had an NF1 family history. At diagnosis, cafe-au-lait spots were present on nine patients, and freckling was recorded in one. Lisch nodules were not detected in any patient. Six patients developed neurofibromas. With regard to the associated clinical conditions, short stature was reported in two patients, and tibial pseudarthrosis and clitoris hypertrophy, respectively, in one. One patient presented with

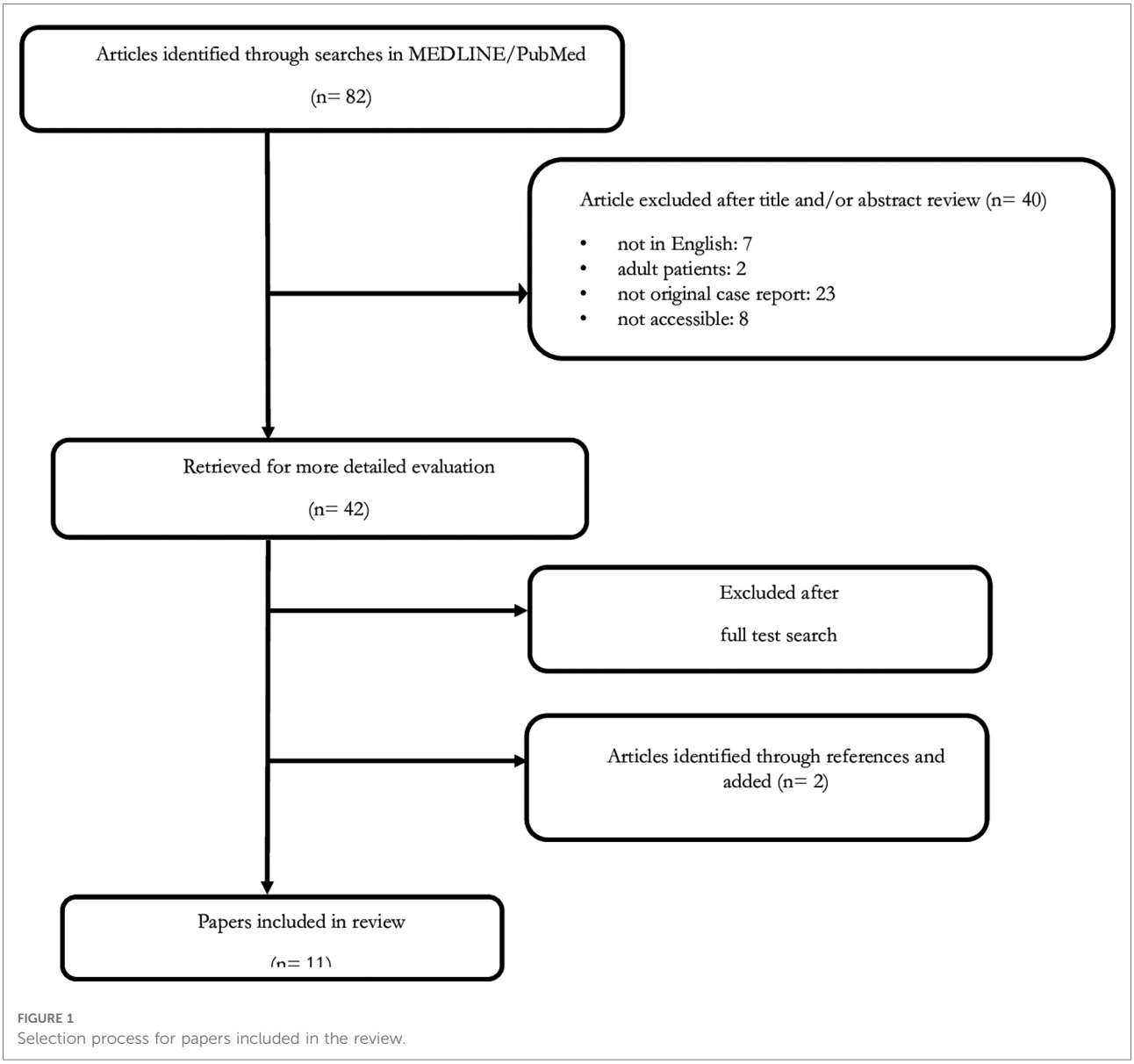

FIGURE 1
Selection process for papers included in the review.

macroglossia, laryngomalacia, Meckel's diverticulum, and esophageal gastric heterotopia. Two cases of schwannoma and one case of optic glioma were registered. Macrocephaly was not described. With regard to the cognitive and behavioral profile, only one case of retarded psychomotor development was reported. Genetic testing was performed in two patients.

A diagnostic biopsy was performed on four patients, two for primary and two for liver metastases, while a major resection was obtained in four patients, in one, together with laminotomy; in one patient, diagnosis was made by using a bone marrow aspirate and MIBG scintigraphy and in one patient after autopsy.

Among 11 patients whose treatment was reported, three received only chemotherapy during the first-line treatment and four both chemotherapy and radiotherapy. For patients with GN and GNB, only surgery was the first-line treatment. Patient No. 4 received chemotherapy, radiotherapy, retinoic acid, and therapeutic MIBG at relapse. Patient No. 1 was the only one who received, in addition to chemotherapy during the first-line treatment, megatherapy with stem cell rescue.

The outcome was not reported for two patients. Four patients survived after first-line therapy, in CR, but only one maintained CR and was described as "alive" 12 months after diagnosis, while the other three eventually relapsed and died of the disease. Two patients obtained a PR, but only one is now alive in CR, while the other relapsed and died of NB. The remaining four patients showed rapid progress but eventually died of NB. The median age at death was 49.5

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TABLE 5 Demographic information of the case reports.

Characteristic	Reported only on 10 patients
Sex, No. Male	7
Female	3
Not available	2
Age in months at diagnosis of NBT	
Mean	39.5
Median	27
Range	0–168
Age in months at diagnosis of NF1 (evaluated on nine patients)	
Mean	23.1
Median	14
Range	2–72
Age at death	
Mean	76.3
Median	49.5
Range	2.4–174
Survival in months after diagnosis	
Mean	38.8
Median	21.75
Range	0–120

NBT, neuroblastic tumor.

months (range, 2–174 months), with a median survival of 21.75 months after diagnosis.

To the best of our knowledge, this is the first study on the spectrum of NBT in Italian patients with NF1, which is one of the cancer-predisposing genetic disorders. To provide additional information, we reviewed the clinical and survival data of children with NBT and NF1 enrolled in the RINB over a 38-year period.

The frequencies of benign and malignant tumor development are increased in children with NF1 compared with the healthy population. In a recent study, it was found that the incidence of malignancy in children with NF1 younger than 16 years was 14.7% (39). Our report confirms that NBTs are very rare in patients with NF1 (only 0.2% of all patients registered in the RINB). In most NF1-related malignancies, including astrocytomas, MPNSTs, and neuroblastomas, a biallelic mutation of the *NF1* gene function is found in the affected cells (40–43) demonstrated homozygous inactivation of the *NF1* gene found in NB cells of a patient affected by familial NF1 and stage 4 NB. It is known that patients with stage 4 NB display an aggressive behavior, which is possibly connected with genetic abnormalities like *MYCN* gene amplification, chromosome 1p36 deletion, and chromosome 17q gain (44). Furthermore, an analysis of the *NF1* gene in NB cells revealed a somatic paternal allelic deletion encompassing the introns 26 and 27b and a maternal-derived constitutional T → C transition in the donor splice site of intron 14. Martinsson et al. (43) described a homozygous deletion of the *NF1* gene in a patient affected by NB. This patient showed a large biallelic deletion of *NF1* in the tumor cells that also revealed chromosome 1p36 deletion but not *MYCN* amplification (43).

Although this particular patient had a prognostically favorable localized disease (stage 2), his tumor underwent progression, leading (like in our own case) to his death. This suggests that the mutation of the *NF1* gene may be associated with a more aggressive tumor behavior. Hence, homozygous inactivation of the *NF1* gene could result in a partial or total abrogation of neurofibromin activity, which would lead to increased intracellular RAS signaling; this could lead to abnormal cell proliferation. A maternal or paternal germline mutation associated with a paternal or maternal somatic deletion found in NB cells indicate that an inactivation of the *NF1* gene in tumor cells occurred according to the "two-hit" model. Moreover, this mutation could be also inherited in a *de novo* way. The two-hit hypothesis has also been suggested by Martinsson et al. to explain the homozygous inactivation of the *NF1* gene. A somatic inactivation of the still functioning *NF1* allele is believed to be required for tumor formation. This "second hit" creates a reduced function of neurofibromin in the affected cells, diminishing its normal functions, including those of controlling cell growth and proliferation. With this role of the *NF1* gene as a tumor suppressor, it is not surprising that somatic mutations of the *NF1* gene are also commonly found in different non-NF1-associated tumors. Both patients died of tumor progression, even though one had favorable clinical characteristics, suggesting that the biallelic inactivation of *NF1* might induce a more aggressive growth behavior of the disease.

Both in our study and in our review, the media and median age at diagnosis of neuroblastoma are similar, which is in agreement with the data that 95% of all neuroblastomas occur in children under 5 years of age. However, there is a difference in terms of the male-to-female ratio. In the review study, there is a prevalence of males, which is in concordance with the majority of reviewed studies in which neuroblastoma is found to be slightly more common among boys than among girls. However, the cases analyzed in the Italian study show a slightly greater frequency in females. This is in agreement with some studies that reported that females with NF1 had a higher risk of malignancy than males (26), but we did not replicate this observation, which may be attributed to our relatively small sample size limiting the statistical power to detect a difference.

Neuroblastomas have a very broad spectrum of clinical behavior, which can include spontaneous regression, maturation to a benign GN, or aggressive disease, with metastatic dissemination leading to death. This pattern of malignancies was similar to what we found in our NF1 patients and included a variety of different histotypes with a

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TABLE 6A Description of the cases reports analyzed by the literature (part 1).

Patient No.	1	2	3	4	5	6
Author, tear	Cheuk, 2013	Duhem-Tonnelle, 2006	Origone, 2003	Martinsson, 1997	Hayflick, 1990	Qualman, 1986
Year	2013	2006	2003	1997	1990	1986
Sex	Male	Male	Male	Unknown	Male	Female
Age at diagnosis of NBT (months)	19	22	36	72	9	2 (autopsy)
Age at diagnosis of NF1 (months)	unknown	unknown	36	72	9	2 (autopsy)
NF1 family history	unknown	Positive (not specified)	mother and brother	Father	father	No
NF1 features described	unknown	CAL	CAL	CAL	CAL, NF	CAL, NF
NF1 gene analysis	Unknown	Unknown	Positive	Positive	Unknown	Unknown
Symptoms at diagnosis	Supraclavicular lymphadenopathy hepatomegaly	Progressive tetraparesis associated with cervical pain	Paleness, periorbital ecchymosis, and a large abdominal mass	Unknown	Right arm and bilateral lower-extremity weakness edema of the right arm	Apnea, cyanosis
Associated clinical conditions	Unknown	Unknown	tibial pseudoarthrosis	Unknown	short stature, retarded psychomotor development	Schwannoma EEG abnormalities Macroglossia Laryngomalacia Meckel's diverticulum esophageal gastric heterotopia
Primitive tumor site	unknown	Neck	Abdomen (ganglia)	Thorax	Thorax	Abdomen (bilateral adrenal gland)
Histology	NB	GNB	NB	GNB	NB	NB
Diagnosis procedure	Unknown	partial resection with laminotomy	Bone marrow aspiration and MIBG	complete resection	Biopsy	Autopsy
Dumbbell tumor	Unknown	Yes	No	No	Yes	No
INSS	4	Unknown	4	2A	4S	unknown
Metastases	Yes (site no specified)	No	Liver Bone marrow	No at diagnosis Bone and bone marrow at relapse	Lymph nodes Lungs Neck	No
Serum LDH	Unknown	Unknown	elevated	Unknown	Unknown	Unknown
Urine VMA	Unknown	Unknown	elevated	Normal (elevated at relapse)	elevated	Unknown
MYCN status	Unknown	Unknown	Amplified	Unknown	Unknown	Unknown
Surgery first line	Yes	Yes	No	Yes	Yes	No
Chemotherapy first line	Yes	No	Yes	No	Yes	No
Radiotherapy first line	Yes	No	No	No	Unknown	No
MIBG	Unknown	Unknown	Unknown	Unknown	Unknown	No
Response	CR	PR	CR	CR	NR	DP
Relapse/disease progression	Relapsed at 15 months	DP 2 years after diagnosis	Relapsed at 13 months after CR	Relapsed at 2,5 years after CR	Unknown	None
Rescue treatment	Unknown	Surgery	Unknown	S, CT, RT, MIBG, and retinoic acid	Unknown	None
Outcome	Died at 39 months for DP (20 months after diagnosis)	Died at 108 months for respiratory failure (84 months after diagnosis)	Died at 60 months (26 months after diagnosis)	Died at 168 months (96 months after diagnosis)	Unknown	Died at 2 months for disease (aspiration pneumonia and partial airway obstruction)

CAL, cafè-au-lait spot; CR, complete response; CT, chemotherapy; DP, disease progression.; GNB, ganglioneuroblastoma; INSS, International Neuroblastoma Staging System; LDH, lactate dehydrogenase; MIBG, 131I-metaiodobenzylguanidine; NB, neuroblastoma; NBT, neuroblastic tumor; NF neurofibroma; NR not reported; PR, partial response; RT, radiotherapy; S, surgery; VMA, vanillylmandelic acid.

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TABLE 6B Description of the cases reports analyzed by the literature (part 2).

Patient No.	7	8	9	10	11	12
Author, year	Qualman, 1986	Kushner, 1985	Nakagawara, 1985	Weiner, 1982	Witzleben, 1974	Knudson, 1966
Year	1986	1985	1985	1982	1974	1966
Sex	Male	Unknown	Female	Male	Male	Female
Age at diagnosis of NBT (months)	6	54	168	54	32	0
Age at diagnosis of NF1 (months)	3	24	14	NR	12	36
NF1 family history	No	Mother	Brother	Unknown	mother, father	No
NF1 features described	NF	CAL, freckling	CAL, NF	Unknown	CAL, NF	CAL, NF
NF1 gene analysis	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Symptoms at diagnosis	Abdominal masses and nasopharyngeal tumor	Fever Leg pains	Fever, abdominal mass, weight loss, ptosis	Fatigue, epistaxis, leg pains, hepatosplenomegaly, lymphadenopathy	Lethargy, irritability, somnolence, cachectic, abdominal mass	Persistent vomiting
Associated clinical conditions	Schwannoma NF	Unknown	Malignant pheochromocytoma	LLA	Short stature, Optic glioma	Clitoris hypertrophy, Horner's syndrome
Primitive tumor site	Thorax, abdomen	Abdomen (adrenal gland)	Abdomen (adrenal gland)	Thorax	Thorax	Thorax
Histology	NB	NB	NB	GN	NB	NB and GN
Diagnosis procedure	Biopsy	unknown	Biopsy (liver metastasis)	Resection	Resection	Biopsy (liver metastasis)
Dumbbell tumor	Unknown	Unknown	Unknown	No	No	unknown
INSS	4	4	4	NA	4	4s
Metastases	Lungs, prostate	Bone, bone marrow	Liver, bone, lungs, lymph nodes, bone marrow	Unknown	Bone marrow, liver, lungs, lymph nodes, bone	Liver
Serum LDH	Unknown	Unknown	Elevated	Unknown	Unknown	Unknown
Urine VMA	Normal	Unknown	Elevated	Normal	Unknown	Unknown
MYCN status	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Surgery first line	Yes	Unknown	Yes	Yes	Yes	Yes
Chemotherapy first line	Yes	Unknown	Yes	No	Yes	Yes
Radiotherapy first line	Yes	Unknown	Unknown	Unknown	Yes	Yes
MIBG	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Cis-retinoic acid	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Response	DP	NR	DP	CR	DP	PR
Relapse/disease progression	Unknown	Unknown	Unknown	Unknown	Unknown	No
Rescue treatment	Unknown	Unknown	Unknown	Unknown	unknown	No
Outcome	Died at 26 months (23,5 months after diagnosis) for progressive airways obstruction and portal hypertension	unknown	Died at 174 months (6 months after the diagnosis)	Alive at 12 months after diagnosis	Died at 33 months (1 month after the diagnosis)	Alive in CR at 120 months from diagnosis

CAL, cafè-au-lait spot; CR, complete response; DP, disease progression.; GN, ganglioneuroma; INSS, International Neuroblastoma Staging System; LDH, lactate dehydrogenase; MIBG, 131I-metaiodobenzylguanidine; NA, not applicable; NB, neuroblastoma; NF neurofibroma; PR, partial response; VMA, vanillylmandelic acid.

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general prevalence of neuroblastoma on ganglioneuroblastoma and ganglioneuroma. Diagnostic dilemmas may arise in patients with NF1 and malignancy because neurofibroma and neuroblastoma share a common origin in the neural crest. Both show rapid growth and extensive invasion. Diagnosis is further complicated by the potential for different histologic components existing within one tumor mass. The biopsy of a portion of a heterogeneous tumor may not be representative of the spectrum of pathology.

Adrenal glands were the most common primary tumor site in our series and presented as an abdominal mass with symptoms of compression of the abdominal viscera. This finding was consistent with the findings of previous studies, followed by retroperitoneal and pelvic sympathetic ganglia (**Figure 2**). Thoracic localization was reported in cases studied in the literature, but not in ours. The SEER monograph reported that, regardless of age, neuroblastomas most frequently occurred in the adrenal gland (45).

The benefits of early diagnosis are well documented and include monitoring for malignancy. A possible pathogenic relationship between NF1 and NBT is based on several observations. Neurofibromatosis is a neurocristopathy. The clinical features are pathogenetically united in their origin in neural crest dysgenesis. NBTs arise from cells of the neural crest as well. INSS stage 4s neuroblastoma is characterized by a small primary tumor in infants less than 12 months old in whom remote involvement is confined to the liver, skin, or bone marrow. The tumor can regress to lesions that are pathogenetically indistinguishable from neurofibroma. Early in its development, neurofibroma may cytologically resemble a differentiating neuroblastoma (46). In children, an extremely rapid growth of a neurofibroma is uncharacteristic and warrants investigation for malignancy. In addition to physical examination, imaging studies and urinary catecholamine measurements are indicated. Because management decisions depend on distinguishing malignant from non-malignant tumor, direct tissue diagnosis of both neuroblastoma and neurofibroma is essential.

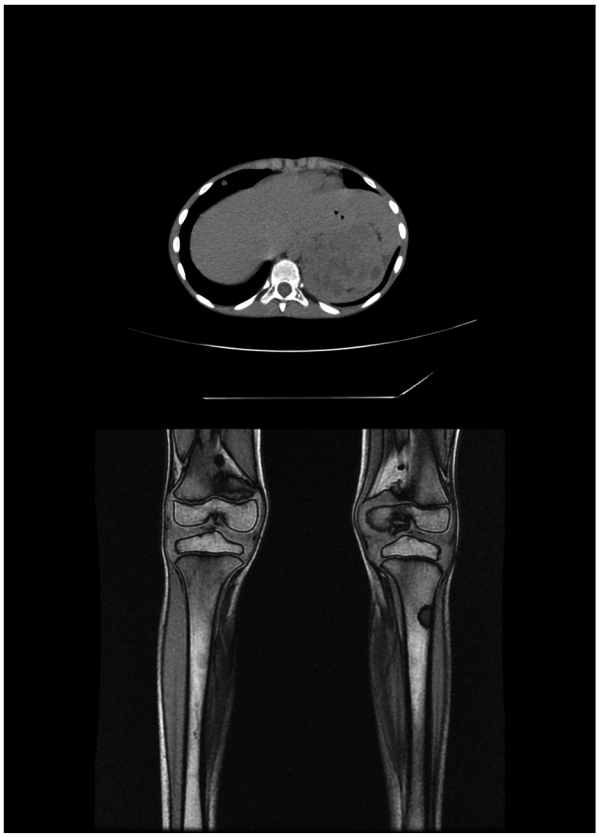

FIGURE 2
Patient No. 3's MRI images. MRI, magnetic resonance imaging.

In the present study, the staging distribution is fairly heterogeneous, with the majority of patients with an INSS stage 4 neuroblastoma. However, it is interesting to note how, compared with the review, the percentage of metastatic tumors has decreased over time. The high percentage of stage 4 in the review study may be attributed to a lack of awareness shown by general practitioners of the probability of cancer, especially in infancy when localized stages are more common, and to the lack of ultrasound use, such that tumors were not initially identified and subsequently regressed or later diagnosed at a more advanced stage in a tertiary hospital. This explanation may also account for similar findings in the postulation by Spix et al., in a study of neuroblastoma in Europe between 1978 and 1992, that the variation in stage distribution between countries may be explained by differences in the frequency of diagnosis of localized cases (5).

In our study, as described by the INSS, the rate of patients with elevated VMA and LDH at diagnosis varies according to the stage, with high-stage tumors being more likely to have pathological values. *MYCN* amplification, performed in five tested tumors, was absent, while the literature refers to only one case in 2003 with amplified *MYCN* (42).

In general, the treatment of neuroblastoma in NF1 patients is similar to that in patients without NF1. When dealing with biopsy specimens, the main concern is related to arriving at a diagnosis. The material should, therefore, be subdivided into at least two parts: one for diagnosis and the definition of cellular composition and the other for touch preparations and molecular biology investigations. In our series, only one patient (No. 4) did not undergo surgery, and diagnosis was made by using bone marrow aspiration; on the remaining patients, biopsy was performed. In our series, multiple-agent chemotherapy was adopted systematically and judged, and it was preferable to invasive surgery as an initial approach. This had numerous advantages for the patients, because no major surgical complications were reported and tumor shrinkage after chemotherapy allowed delayed surgery to be less

aggressive. Unfortunately, complete tumor resection could be still difficult to achieve, but our chemotherapy and radiotherapy may be enough to control postoperative residuals, and some patients were cured without having to undergo any major surgery.

An interesting presentation is that of one of our patients (patient No. 5), who showed the most significant number of complications related to NF1, with bilateral hydronephrosis (with nephrostomies), clitoral hypertrophy, pelvic plexiform neurofibromas, neurofibromas with invasion of the spinal canal and compression of the spinal cord, and MPNST. This patient developed a ganglioneuroma, which, as already mentioned, is considered benign (47) and the prognosis is excellent, even when complete tumor removal is not possible. Nevertheless, the patient had a pelvic mass infiltrating the uterus, urethra, and sciatic nerve, with the nodules reaching the right gluteus. The histological examination carried out following biopsy revealed a diagnosis of plexiform neurofibromas with ganglioneuroma areas. Given the extent of the disease and the infiltration of the pelvic organs, surgical treatment was excluded, and it was decided to perform a strict radiological clinical follow-up. Because of the dimensional increase in the mass, the appearance of pain, and the accentuation of itching, the patient was subjected to metabolic radiotherapy with MIBG, given the intense capture of the documented mass with MIBG scintigraphy. After two cycles with no response, attempts were made with different chemotherapy cycles, first with cisplatin and etoposide, then with cyclophosphamide, and finally with methotrexate and vinorelbine. Finally, the patient showed a steady state of the mass with the administration of lenalidomide. Unfortunately, however, the patient died subsequently following the development of a mediastinal MPNST. This presentation should be regarded as an exception.

The analysis of our series of cases shows a death outcome in half of the patients. In the literature review, only two of ten patients reported survived after treatment.

The results indicate that the survival rates reported in our series are higher than those described in previously published reports. For this, many explanations should be considered: the small number of patients in both series, that is, in ours and in the literature; most of our patients have been diagnosed later than those in the literature. Moreover, this may be due to the systematic use of a multidisciplinary approach in all the patients concerned and to the ongoing trials that are exploring the efficacy of new drugs and novel immunological approaches in order to save a greater number of these patients. Unfortunately, the need for an aggressive treatment approach can also mean severe side effects, and this issue should be addressed when planning future treatments.

The current study has several limitations. It is a retrospective study. Compared with the reported national studies in other countries, the relatively small sample size in ours was an important limitation affecting statistical power. However, this is already the largest study on neuroblastoma in patients with NF1, and considering the fact that we do not have a registry on malignancies in NF1 patients, these are the best currently available data.

Undoubtedly, case reports will not have as much potential impact on the science or practice of healthcare as a randomized controlled trial or other research projects on cancerous diseases. However, case reports have a role to play only in evidence-based medicine and constitute the first line of evidence, also considering that in cancer therapies, the number of patients required to conduct such studies cannot always be recruited. It may be the only way to make others in the field aware of unusual presentations or complications, and it is a time-honored vehicle for teaching others.

Data availability statement

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

Ethics statement

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the 2016/2018 Research Plan of the University of Catania, Department of Clinical and Experimental Medicine (project #B: "Evaluation of long-term harmful effects of chemotherapy and radiotherapy on various organs in childhood cancer survivors").

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The reviewer ADP declared a shared affiliation with the author(s) FP, RS, MM, GR, AP, MR and ADC to the handling editor at the time of review.

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frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 15 November 2022
DOI 10.3389/fonc.2022.994934

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 15 July 2022
ACCEPTED 12 October 2022
PUBLISHED 15 November 2022

CITATION
Burgers VWG, van den Bent MJ, Dirven L, Lalisang RI, Tromp JM, Compter A, Kouwenhoven M, Bos MEMM, de Langen A, Reuvers MJP, Franssen SA, Frissen SAMM, Harthoorn NCGL, Dickhout A, Noordhoek MJ, van der Graaf WTA and Husson O (2022) "Finding my way in a maze while the clock is ticking": The daily life challenges of adolescents and young adults with an uncertain or poor cancer prognosis.
Front. Oncol. 12:994934.
doi: 10.3389/fonc.2022.994934

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"Finding my way in a maze while the clock is ticking": The daily life challenges of adolescents and young adults with an uncertain or poor cancer prognosis

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Introduction: Increasingly more adolescent and young adult (AYA, aged 18–39 years) patients with an uncertain and/or poor cancer prognosis (UPCP) are gaining life-years because of novel treatments or refinement of established therapies, and sometimes even face the prospect of long-term disease control. This study aims to examine the challenges of AYAs with a UPCP in daily life to inform the development of AYA care programs.

Methods: Semi-structured in-depth interviews were conducted among AYAs with a UPCP. Since we expected differences in experiences between three AYA subgroups, we interviewed patients of these subgroups (1): traditional survivors (2), low-grade glioma survivors, and (3) new survivors. Interviews were analyzed

using elements of grounded theory. AYA patients were actively involved as research partners.

Results: In total 46 AYAs with UPCP participated and shared their challenges in daily life. They were on average 33.4 years old (age range 23–44) and most of them were women (63%). The most common tumor types were low-grade gliomas (16), sarcomas (7), breast cancers (6), and lung cancers (6). We identified five primary themes: (1) feeling inferior to previous self and others (e.g. feeling useless, who wants me in a relationship), (2) feeling of being alone (e.g. lonely thoughts, nobody really gets me), (3) ongoing confrontation (e.g. it is always there, own decline), (4) grief about life (e.g. grief about life I did not get, grief about old life), and (5) loss of control over the future (e.g. not able to make future plans, waiting for growth). Although all of the challenges were identified in the three AYA subgroups, the perceived intensity of the challenges differed slightly between the subgroups.

Discussion: AYAs living with a UPCP experience challenges associated to their sense of altered identity, their position in the social network, and the future uncertainties. This study highlights the importance to recognize and acknowledge the unique challenges of this group. To provide age-specific care, it is important to embed acceptance and commitment therapy and AYA peer support within the healthcare system and other care programs to support AYAs to live well with their disease.

KEYWORDS

adolescents and young adults, poor or uncertain cancer prognosis, psychosocial challenges, psychological therapy, qualitative research

Introduction

An important but often overlooked group of cancer patients are adolescents and young adults (AYA; 15–39 years of age) with an uncertain or poor cancer prognosis (UPCP). Up to now, most psychosocial research studies in this age group have been focused on patients with a curative intent, and more recently, attention is paid on those in the end-of-life phase (1, 2). However, due to advances in treatments, a growing number of AYAs with a UPCP are living longer with cancer (3, 4). We recently defined AYAs with a UPCP as those with advanced cancer for which there is no reasonable hope of cure, indicating that they will die prematurely from cancer, but have no immediate threat of death (5). Patients with terminal illness, defined as those with a life expectancy less than 3–6 months and poor performance status, do not belong to this group (5, 6). Patients with a UPCP were classified into three groups based on received treatment and prognosis, i.e. those (1) treated with standard established treatments (e.g. chemotherapy; traditional survivors with a life expectancy of 1–5 years) (2), undergoing novel treatment (s) (e.g. targeted therapy and immunotherapy; new survivors with uncertain prognosis), or (3) with a low-grade glioma who are living with the knowledge that tumor progression inevitably occurs and likely will be lethal (5).

The diagnosis of advanced cancer in adolescence or young adulthood can bring one's life and the achievement of milestones to an abrupt halt. The attention of an AYA has to shift to cancer-related challenges including management of side effects, dealing with the threat of death and the future uncertainties; instead of developing and realizing relationships, careers, and achieving future goals (7–9). AYAs struggle with lost opportunities to participate in a long, healthy, and meaningful life, while most of them do not yet have the coping skills to deal with this situation (10). These age-related challenges make AYAs with a UPCP a vulnerable group. However, these challenges have not yet been well studied since this patient group is often "absorbed" in the large group of AYAs treated with curative intent with an overall good prognosis, in the overall adult cancer population or in the group of patients near the end of life (11). The limited qualitative research on AYAs with advanced or metastatic cancer report that AYAs experience a sense of isolation from healthy peers, AYA cancer patients with curative treatment goals, and older

patients (12). The loss of control over their life and the psychological challenge of anticipatory grief over the life that has not yet been lived might be difficult for AYAs to process (10, 11, 13). Due to the prognostic uncertainty, AYAs are constantly balancing between hope and fear amidst uncertain treatment outcomes (14).

According to a meta-review study of Garcia et al., patients living with advanced cancer in general experience situations or events that involve suffering (e.g. physical symptoms, spiritual and psychological suffering) (15). Patients with advanced disease try to reduce this suffering and find new meaning in life through coping mechanisms, such as adapting to change and keeping hope. Garcia et al. identified the common desire of patients to preserve or reclaim normalcy by engaging in usual daily activities. However, this can be difficult to pursue due to physical, psychosocial, and emotional challenges (15–19). Challenges for AYA with a UPCP might be even more profound due to their unique and uncertain disease situation while being in a vulnerable developmental life phase. Therefore, the aims of this study were to examine more specifically the psychosocial challenges in daily life of the growing group of AYAs with a UPCP.

Methods

Study design

A qualitative study was conducted. Elements of the Grounded Theory by Corbin and Strauss were used in combination with a constructivist philosophical perspective (20). This perspective implies the acknowledgement of (1) the existence of multiple complex realities that relate to real events, (2) differences in participants' responses to events, and (3) that the construction of theories is dependent of the researchers (20–22). This approach was chosen because of the ability to understand the actions of individuals in a context of their unique life-stage and diagnosis while facing problems or specific social situations. To ensure good quality of our study, the 'Consolidation Criteria for Reporting Qualitative Studies' (COREQ) guidelines were followed (23) (Supplementary Material 1).

Participants

Patients were recruited in eight University Medical Centers in the Netherlands, the Netherlands Cancer Institute, and one large nonacademic teaching hospital (Haaglanden Medical Center). Eligible patients included those (1) diagnosed with any type of advanced cancer for the first time between 18 and 39 year of age, for which there is no reasonable hope of cure, indicating that the patient will die prematurely due to cancer, and (2) able to speak and understand Dutch. The age range between 18 and 39 years was chosen because this is the age definition of AYA cancer patients in the Netherlands. Patients with terminal illness with a life expectancy of 3–6 months or less and a poor performance status were excluded. This decision was based on the prognostic estimates of the treating clinician *via* a surprise question ("Would you be surprised if this patient died within the next 6 months?"). Patients with significant cognitive problems, who were not able to complete an interview as determined by the treating clinician, were also excluded.

Potential patients meeting the eligibility criteria were identified and informed by their treating clinician *via* telephone or face-to-face in clinic. After obtaining permission, the researcher contacted the patient to provide further information and obtained written informed consent. Since we expected differences in experiences between the three AYA subgroups described above (traditional survivors, new survivors, low-grade glioma survivors), we aimed to interview at least 16 patients per distinct subgroup to ensure a heterogeneous sample. The research team (VB, OH, WvdG) divided patients in the three subgroups based on information from the treating clinician regarding the received treatment and prognosis (5). We applied a purposive sampling strategy, aiming to achieve good representation of sex/gender, age, and tumor types within each subgroup. The minimum of 16 patients per subgroup was a starting point for the recruitment phase to cover the full spectrum of the diverse group of AYAs with a UPCP. Additionally, we included five eligible AYA patients (divided in the three subgroups) in a focus group, which is described in more detail in the next section. Data collection stopped when saturation was reached, next to the minimum predefined sample size, and the researcher had a clear overview of the challenges of AYAs with a UPCP experienced in daily life. Ethical approval was given by the Institutional Review Board of The Netherlands Cancer Institute (IRBd20-205). In the other participating centers, this study was approved by their ethical committees according to local regulations.

Procedure

Interviews were conducted between April 2021 and April 2022 during the COVID-19 pandemic. A trained female psychologist and qualitative researcher (VB) conducted the semi-structured in-depth interviews *via* video calls. An interview guide with open questions and probes was created based on literature. This interview guide was drafted in collaboration with experienced researchers (WvdG, OH) and AYA patients who are actively involved as research partners in this study (AD, NH, MN, SF, SF). In order to optimize the transparent reporting of the active involvement of patients as research partners in this study, the GRIPP2-SF checklist was followed and completed (24) (Supplementary Material 2). The

AYA research partners reviewed and adapted the interview guide for relevance, comprehensiveness, word use, and level of confrontation. The interview guide was pilot-tested and minor adjustments were done twice during the data collection to obtain the most optimal information (Table 1). Interviews were audio-recorded and lasted 84 min on average (range: 49–122). Notes and summaries were made to provide context for analysis. Each participant was called a week after the interview to evaluate their experience with the interview and to provide an option to share additional relevant information that was not covered during the interview.

The preliminary results of the study were shared with the AYA research partners and discussed during focus groups with AYA research partners. The focus group aimed to check for interpretation and correctness of a patients' point of view. Since the focus group discussions gave also additional insights into the daily challenges of AYAs with a UPCP, we decided to incorporate these findings into the main results of this study. The AYA research partners all provided informed consent to be included as participants in this study. In total, five focus groups were conducted by VB, all were audio-recorded and lasted 67 min on average (range: 53–90).

In addition to these interviews and focus groups, patients completed a short case report form (CRF) on their sociodemographic and medical characteristics including; age, gender, ethnical and religious background, level of education, employment status, living and care arrangements, age on diagnosis, and comorbidity using Charlson Index. Clinical data, including primary diagnosis, date of diagnosis, stage of disease, and type of treatment (if any) were obtained from the treating clinician after patients gave permission for requesting medical data.

Data analysis

Interviews and focus groups were transcribed verbatim and the transcripts were analyzed by VB using elements of the grounded theory by Corbin and Strauss (20). Analysis started immediately after the first interview. A cyclic process was applied of interviewing, visualizing the interview, analyzing, and reflection by memo writing, leading to new questions and more interviews. Analysis started with open coding and axial coding with the help of NVivo (25). For the first 15 interviews (five of each subgroup), another independent researcher (MR) repeated this process, in order to identify and discuss different perspectives on the same data. In case of disagreement, the remaining codes were discussed with the AYA research partners and the research team. In the meetings with the AYA research patients and research team, everyone was equal in the decision-making process. Open coding and axial coding were done concurrently. Axial coding was done with the help of "the paradigm' of Corbin and Strauss, an analytical tool to structure around a category in terms of conditions, actions–interactions, and consequences (20). These steps were followed for each unique interview in which each new interview was compared with the data of the other interviews. VB wrote memos, used strategies like asking questions, constant comparison, and finding the negative case(s) to understand the essence of what was being said: the underlying issue of the

TABLE 1 Interview topic guide.

Questions	Probes
Could you please tell me how your disease (or preferred term by AYA) has affected your life?	Could you please tell me how your disease (or preferred term by AYA) has affected your daily life?
	Could you please tell me how your disease (or preferred term by AYA) has affected important life choices? (e.g. study, job, children)
	Could you please take me through a specific case which describes your experience in the best way?
	Can you give an example that visualizes the challenges you came across when dealing with your disease?
	Do you think the impact would have been different when you know for sure that you will be cured?
Could you please tell me how your disease has affected your relationship with your loved ones?	Could you please give me an example that showcases this well?
	Who of you is changed? Could you please elaborate?
How do you experience the support you receive?	Could you please elaborate? (from who, how and what frequency do you experience support)
	What kind of advice would you give to your support system?
	What is the best way to support you right now?
	Do you miss or have missed some kind of support?
What are your thoughts about the future?	How is your future perspective changed after your diagnosis? And has this changed over time?
	What concerns you the most at the moment? What are you afraid of?
	Do you already have taken action regarding your future, which you never would have done on this point in your life if you were not diagnosed with this disease?
	What are your needs regarding communication about the future?

challenges AYAs experienced. After analyzing half and after analyzing all of the interviews, the codes were checked by the AYA research partners as a form of member checking. The focus groups were analyzed the same way as the interviews and the interpretation of the results were checked by the AYA research partners themselves. With selective coding, all the data came together to construct a plausible explanatory framework about the challenges of living with a UPCP at AYA age. This general framework was checked for misinterpretation or gaps in logic by the research team and AYA research partners, resulting in minor adjustments. Data collection stopped when conceptual saturation was reached (20).

Results

In total, 64 patients were invited, of whom 46 (72%) were actually interviewed. Eleven patients declined due to illness, four patients were too busy or did not want to focus on their disease, and three patients did not respond. Due to a combination of reaching conceptual saturation and repeated illness of the last patients that needed to be included, we ended up with 15 patients in two subgroups and 16 in the low-grade glioma subgroup. The mean age of AYAs at time of the interview was 33.4 years (Table 2). The majority of the interview and focus group participants were women, had a Dutch ethnicity, were married or had a partner and did not have children (Table 2). A range of tumor types were included, most commonly (low-grade) gliomas, followed by sarcomas, breast cancers, and lung cancers.

We identified five main themes in which we incorporated the influence of the COVID-19 pandemic according to the experiences of the AYAs with a UPCP. The themes, subthemes, and codes along with quotes are presented in Table 3. Numbers in parentheses denote the in-text reference for the quote in the table. A visual overview of the results is presented in Figure 1. Table 4 provides a complete overview of differences between the AYA subgroups (Table 4).

Theme 1. Feeling inferior to previous self and others

1.1 Other identity

The physical, mental, and psychosocial (long-term) challenges that come together with an advanced cancer diagnosis require from AYAs to find a new balance in life and ask for an adjusted identity. AYAs with a UPCP relate their identity specifically to work or education. Adjustments to work life or education (e.g. stop working, working fewer hours) create feelings in AYAs as if they are not useful, like they achieved nothing or as if they lost the connection to society, leading to diminished self-esteem (1.1.1/1.1.2). Adaptions in work life were in general more present due to COVID-19 and the feelings as described above were especially the case in AYAs with a low-grade glioma. Since AYAs did not want to be identified as patients; some of them have the urge to prove themselves at work, especially when they have the idea to be treated differently by colleagues or employer due to their cancer (1.1.3). Younger patients wanted to identify themselves as an excellent patient, who is able to finish their study to show that they are no quitters and stay part of the society.

In addition, the uncertain future leads some AYAs to anxiety around managing a work task or returning to work due to concerns that they might not have the physical or cognitive ability to do so. Additionally, AYAs relate their identity to their role as a friend and parent (1.1.4). All together, these experiences make AYAs feel inferior to their previous self and others, which was mostly the case in AYAs with low-grade glioma and traditional survivors.

1.2 Who wants a romantic relationship with me?

AYAs experience challenges forming new romantic relationships as they are feeling inferior to the other and feel guilty to hurt another because of their cancer and potential premature death (1.2.1). Afraid to be rejected because of their cancer diagnosis, some AYAs are struggling with finding the right moment to share their cancer story while dating and others totally avoid dating (1.2.2). AYAs with a relationship experienced a sense of guilt for their loved ones because of the extra practical burden on the shoulders of their partner, the tension of the situation, and the perceived inequality as they feel they cannot give their partner what he or she deserves (1.2.3). Because of the uncertain prognosis in a life stage full of milestones, existential questions emerged, like 'Is it fair that he cannot become a father because of me?' AYAs reconsider their relationship and some of them ask their partners whether they want to continue their relationship to provide them with a way out (1.2.4).

Theme 2. Feeling of being alone

2.1 Protecting others' feelings

AYAs do have a strong need to protect feelings of loved ones by not sharing everything, not telling all the details, or toning down bad news (2.1.1). AYAs do not want to be a burden and described feelings of guilt when confronted with the sadness of their loved ones, especially parents who are more often quick to switch to panic mode. Even though they know they cannot do anything about it, they still feel like they are the cause of all problems (2.1.2). Not sharing everything results in feelings of being alone.

2.2 Lonely thoughts

Some AYAs are not able to discuss everything about the cancer with their loved ones, especially about end of life. As some AYAs avoid specific topics themselves (2.2.1), others

10.3389/fonc.2022.994934

TABLE 2 Demographics AYA respondents and focus group of AYA research partners.

Characteristics	Total AYAs[a] N (%)	Traditional Survivors N (%)	New Survivors N (%)	LGG survivors N (%)	Focus group N (%)
Age at initial diagnosis, years					
Range	23–39	23–37	22–38	22–39	20–38
Mean (SD)	29.6 (4.8)	28.93 (3.9)	30.7 (4.9)	29.8 (5.7)	27.6 (6.6)
Current age, years					
Range	23-44	24-44	23-41	23-43	23-38
Mean (SD)	33.4 (6.3)	33.0 (5.6)	34.2 (4.8)	33.2 (5.6)	31.8 (5.5)
Gender					
Woman	29 (63.0)	12 (80.0)	10 (66.7)	7 (43.8)	4 (80.0)
Man	17 (37.0)	3 (20.0)	5 (33.3)	9 (56.3)	1 (20.0)
Ethnicity					
Caucasian	46 (100)	15 (100)	15 (100)	16 (100)	5 (100)
Religion					
No	38 (82.6)	13 (86.7)	13 (86.7)	12 (75.0)	5 (100)
Yes[b]	8 (17.4)	2 (13.3)	2 (13.3)	4 (25.0)	0 (0.0)
Marital status					
Married or partnered	38 (82.6)	11 (73.3)	13 (86.7)	14 (87.5)	3 (60.0)
Single	8 (17.4)	4 (26.7)	2 (13.3)	2 (12.5)	2 (40.0)
Living situation					
Living alone	7 (15.2)	3 (20.0)	3 (20.0)	1 (6.3)	1 (20.0)
Living with partner	16 (34.8)	6 (40.0)	4 (26.7)	6 (37.5)	1 (20.0)
Living with partner and children	16 (34.8)	3 (20.0)	5 (33.3)	8 (50.0)	2 (40.0)
Living with children	3 (6.5)	2 (13.3)	1 (6.7)	0 (0.0)	0 (0.0)
Living with parents	2 (4.3)	1 (6.7)	0 (0.0)	1 (6.3)	0 (0.0)
Other[c]	2 (4.3)	0 (0.0)	2 (13.3)	0 (0.0)	1 (20.0)
Children					
Yes	19 (41.3)	5 (33.3)	6 (40.0)	8 (50.0)	2 (40.0)
No	27 (58.7)	10 (66.7)	8 (60.0)	8 (50.0)	3 (60.0)
Highest achieved level of education					
Secondary education or less	4 (8.7)	0 (0.0)	2 (13.3)	2 (12.6)	1 (20.0)
Secondary vocational education	16 (34.8)	5 (33.3)	4 (26.7)	7 (43.8)	1 (20.0)
Applied university	16 (34.8)	6 (40.0)	5 (33.3)	5 (31.3)	1 (20.0)
University	10 (21.7)	4 (26.7)	4 (26.7)	2 (12.5)	2 (40.0)
Employment status[d]					
Student	3 (6.5)	1 (6.7)	2 (13.3)	0 (0.0)	1 (20.0)
Full-time work	11 (23.9)	4 (26.7)	1 (6.7)	6 (37.5)	1 (20.0)
Part-time work	7 (15.2)	0 (0.0)	4 (26.7)	3 (18.8)	1 (20.0)
Self-employed	2 (4.3)	0 (0.0)	0 (0.0)	1 (6.3)	0 (0.0)
Unemployed	1 (2.2)	0 (0.0)	0 (0.0)	1 (6.3)	0 (0.0)
Homemaker	1 (2.2)	1 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)
On sick-leave/work disabled	23 (50.0)	9 (60.0)	7 (46.7)	6 (37.5)	2 (40.0)
Type of cancer					
(Low-grade) glioma	16 (34.7)	0 (0.0)	0 (0.0)	16 (100)	1 (20.0)
Sarcoma	7 (15.2)	6 (40.0)	1 (6.7)	0 (0.0)	1 (20.0)
Breast cancer	6 (13.0)	3 (20.0)	3 (20.0)	0 (0.0)	1 (20.0)
Lung cancer	6 (13.0)	0 (0.0)	6 (40.0)	0 (0.0)	1 (20.0)
Melanoma	3 (6.5)	0 (0.0)	3 (20.0)	0 (0.0)	0 (0.0)

(Continued)

TABLE 2 Continued

Characteristics	Total AYAs[a] N (%)	Traditional Survivors N (%)	New Survivors N (%)	LGG survivors N (%)	Focus group N (%)
Cervical cancer	2 (4.3)	2 (13.3)	0 (0.0)	0 (0.0)	0 (0.0)
Other[e]	6 (13.0)	4 (26.7)	2 (13.3)	0 (0.0)	1 (20.0)
Stage at time of interview[f]					
II	0 (30.4)	0 (0.0)	0 (0.0)	NA[f]	1 (20.0)
III	1 (3.3)	2 (13.3)	0 (0.0)	NA	0 (0.0)
IV	25 (83.3)	13 (86.7)	12 (80.0)	NA	2 (40.0)
N/A or unknown	4 (13.3)	0 (0.0)	3 (20.0)	NA	2 (40.0)
Current treatment[g]					
None/Active surveillance	16 (34.8)	4 (26.7)	1 (6.7)	11 (68.8)	2 (40.0)
Chemotherapy	14 (30.4)	9 (60.0)	2 (13.3)	4 (25.0)	1 (20.0)
Targeted therapy	10 (21.7)	0 (0.0)	10 (66.7)	0 (0.0)	1 (20.0)
Experimental therapy	3 (6.5)	0 (0.0)	2 (13.3)	1 (6.3)	0 (0.0)
Hormonal therapy	3 (6.5)	2 (1.3)	1 (6.7)	0 (0.0)	1 (20.0)
Immunotherapy	1 (2.2)	1 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)
Radiotherapy	1 (2.2)	1 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)
Comorbidity					
None	34 (74)	11 (73.3)	10 (66.7)	12 (75.0)	5 (100)
One	10 (21.7)	3 (20.0)	4 (26.7)	3 (18.8)	0 (0.0)
Two or more	2 (4.3)	1 (6.7)	0 (0.0)	1 (6.3)	0 (0.0)

[a] Total N = total interviewed AYAs, AYAs participated in focus groups are not included. [b] Yes = Christian, Protestant, Islamic. [c] Other = living with brothers, living with parents and son, living with housemates. [d] Not equal to 100% since some participants were partly disabled and worked part-time for the other part. [e] Other = (1) traditional survivors: colon cancer, epithelioid hemangioendothelioma, neuro-endocrine tumor, ovarian cancer (2) new survivors: gastrointestinal stromal tumor. [f] Low-grade gliomas are grade 2 tumors according to WHO Classification of Tumors of the Central Nervous System. Two patients were initially diagnosed with a low-grade glioma, but at the time of the interview it was progressed to a high-grade glioma grade III/IV. [g] Not equal to 100% due to the combination of treatments.

experience reticence by their loved ones. This reticence of their loved ones is noticeable by the intention to deviate from the subject or by downplaying the severity with comments like 'you are not going to die' (2.2.2). AYAs explain this by the fact that they are a few steps further in this thinking process than their loved ones. Some AYAs express not feeling supported by their partner, as the partner is coping completely differently with the situation or is not able to talk about the cancer. AYAs also do not want to be a burden when initiating a subject. All of this result in these lonely thoughts about cancer, and especially death and dying remain in the mind of the AYAs themselves. It is remarkable that in some cases the COVID-19 pandemic was the reason to discuss this topic.

2.3 Nobody gets me

For AYAs with a UPCP it feels like nobody really understands what they are going through. The complexity of their (rare) tumor combined with the invisibility of living with an uncertain and poor cancer prognosis and the lack of peers in comparable situations results in misunderstanding and a lack of empathy from others. Good days without physical issues can be interpreted by their environment as if the AYA is doing really well, while bad days and mental issues remain unspoken (2.3.1). The biggest misunderstanding is related to good scan results, in which it feels like everyone is relieved, the panic is gone, and the cancer can move to the sideline, while the AYAs themselves do not feel that kind of relief since there is still a threat of death and the stress of a new scan in a short time (2.3.2). This response from the environment results in AYAs struggling to talk about cancer-related issues and some even distance themselves from their family and friends.

2.4 Increasing distance to the social network/ environment

The social environment wants to protect the AYA by not sharing everything, bringing their issues in perspective ('my issue is nothing in comparison with your cancer'), and asking others about the AYA instead of talking to the AYA themselves. The environment makes decisions about what the AYA can and cannot handle, which feels like the AYA is moved to the sideline and makes AYAs think about the value of their relationships (2.4.1). Additionally, the significant differences between the life of the AYAs and their peers result in an ever-increasing distance and confrontation since most of the time they cannot join in the normal AYA age-related conversations about parties, weddings, career, and parenthood, causing social withdrawal in some AYAs (2.4.2).

TABLE 3 Coding hierarchy.

Theme	Sub-theme	Codes	In-text reference	Quotes
1. Feeling inferior to previous self and others	1.1 Other Identity	Feel not useful	1.1.1	I am just failing. [.] I think it is bad to say about myself that I feel inferior to others, but I do not feel equal to others. The fact that have a zero-hour contract for 2-3 days a week and even have to take days of because of hospital appointments. With a zero- hour contract you do not participate in the annual evaluation. I was called a 'temporary employee' in teams for over a year. All that doesn't feel equal to me. (m- melanoma)
		Feel like I have nothing achieved	1.1.2	I want to become a professional. Because I notice so little progress and I already have issues exercising besides my six hours of work, is a scary thought for me. I worry that this is all I will be capable off and I will never make more than 1000 euros per month. That really sucks. (m- low-grade glioma)
		Prove to urge	1.1.3	During my probation period, it turned out that I had a brain tumor, so all my colleagues have a permanent contract and I do not because I am sick. So that already does not feel right, even though I am a really hard worker. (w- low-grade glioma)
		Other me, other friendship?	1.1.4	You make friends when you were a certain person. But I am not really that person anymore, I have changed quite a bit. And that makes me very insecure, will they still like me if I cannot do all of this anymore? (w- breast cancer
	1.2 Who wants me in a romantic relationship?	Not good relationship material	1.2.1	You wish someone the best, I don't think I am the best right now. It feels like lying without me lying. And you know you are consciously are going to hurt someone because you are going to die. I don't want someone on purpose and want to protect this person. (w- focus group)
		Afraid to be rejected	1.2.2	I just removed all dating apps. I thought just leave me alone. But in fact I really want nothing more than a relationship. But I am so afraid. in my head it is always: who wants me? (w- cervical cancer)
		Guilt for loved ones	1.2.3	He has a lot more to do, which is difficult for me. He has to take care for me as well as for the kids and I cannot do many household tasks. I think it is skew. I know there is no other way, but he has a job and when he comes home there is a new to do list for him … while I am at home. So he has a lot of responsibilities. It is more patient-caregiver kind of relationship and that is not how you want it. (w- breast cancer)
		Providing partner a way out	1.2.4	I recently presented him that he have the choice to leave me. I told him "you're 35 years old. If we break up, than you have some extra years to find a new girlfriend and starting a family. In case I live for 5 more years, you are 40 and your change to start a family is relatively smaller. Do you realize that?" (w- osteosarcoma)
2. Feeling of being alone	2.1 Protecting others' feelings	Withholding or weakening information	2.1.1	I actually already knew the scan results and I did not want to make them [parents] sad, so then I thought … well this is a good time to go alone because no one is really allowed to accompanied me [COVID-19 restrictions]. But yes, that was of course very stupid if you know that you will receive such a bad message. (w- low-grade glioma)
		Feelings of guilt	2.1.2	If you have received bad news and tell your family and see how much sad they are. seeing your grandparents cry for the first time makes you feel guilty about it and you don't want to say or do not dare to say it next time. (m- focus group)
	2.2 Lonely thoughts	Avoid topic end of life	2.2.1	When I say it out loud, when I discuss it, it becomes reality. So, I dare not to say things out loud. (w- breast cancer)
		Reticence of loved ones	2.2.2	If I am worried or I am talking about. Or I heard a song on the radio and I think oh that is nice song for my funeral, I get a response like 'You are not going to die at all'. (w- sarcoma))
	2.3 Nobody gets me	Invisible suffering	2.3.1	People do not understand. Because you just look normal on the outside. Yes, I mean when I go to a party there is no one who can see that I am sick on the outside. And they know it, but 'your scans are stable, you can last for so many more years'. I am not someone who shouts it out loud and I also want to have a good time when I go out. But I do miss the understanding, especially from the people you would expect some understanding. It is very difficult for me. (w- melanoma)
		Good scan result	2.3.2	It feels like everyone is so relieved, like you had such good news so now we can put is behind us. Like if I have been cured, as if I am no longer a cancer patient anymore. While in fact I will never been cured and always remain a cancer patient. (w- focus group)
	2.4 Distance with family and friends	Be sidelined	2.4.1	I did not dare to tell you because you are struggling with your brain tumor and I call sick because I am not feeling well after giving birth. So you notice that people hesitate to involve me in their problems, because everything they experience is less important than my brain tumor. That is not how I see or feel it at all. It actually feels like you are no longer involved in things and that creates a certain distance. (w- low-grade glioma)
		Gap between lives	2.4.2	The whole evening they talked about weddings and the wish to have children, while I had a conversation with a cancer peer about funerals yesterday. I no longer feel the connection with my old friends, I cannot talk about the topics anymore because I am in a completely different life phase. It is confronting for me,

(Continued)

TABLE 3 Continued

Theme	Sub-theme	Codes	In-text reference	Quotes
				because I cannot fantasize about these things. And this creates distance, because I cannot participate in their conversations. (w- focus group)
				My housemates are also going out, but they never ask me to go with them because they know that it is not possible. While they can just go out until 5 o'clock and I cannot, I sometimes feel alone. Because while the whole house is gone you already sleep in your room at 11 o'clock. It creates distance with your housemates. (m- focus group)
3. Ongoing confrontation	3.1 It is always there	It is part of me	3.1.1	You wake up with the cancer and you go to bed with it. It is part of my life; I am busy 24/7 with being sick, with the cancer, with peers and comparing with peers. (w- cervical cancer)
		Sword of Damocles	3.1.2	The cancer is on my mind at any time of the day, even when I go to the store with an action for shampoo. I really have trouble with that, I start to panic: 'Do I need 3 shampoos? Am I still alive so I can by 3 shampoos?' And I do not think my life will ever get back to normal. It is in the little things. (w- focus group)
		Medical factors	3.1.3	Going to a hospital is just confronting. Then you are dealing with the cancer: I am sick. And if the hospital appointments becomes more frequently, then it is more confronting for me. (w- lung cancer)
	3.2 Own decline	I cannot deny anymore	3.2.1	I always try to push it away, to do something else. But I notice that this is getting more and more difficult. […] I keep getting worse with all kinds of things, with my memory and the fatigue. (m- low-grade glioma)
				I always think: this is a phase, later I will feel better. And my fear is that I am not feeling better anymore, so this is the best I am going to feel for now and in the future. (w- ovarian cancer)
		What you see vs what I experience	3.2.2	It is difficult to indicate my limits with my cognitive decline. For example, with work meetings, minutes have to be taken and then people expected that I can do that just like everyone else. But that is such a challenge for me. I can explain again that it hard for or I will just do it again. It forces me to either go over my limits again or tell everybody about my cognitive problems. (w- focus group)
	3.3 Social world	Stigma cancer patient	3.3.1	My mother was at my place again 'I will do your laundry and then I will do the dishwasher and…'. And I think, I can do all of this myself and I am not totally incapable. People want to do this for you since they cannot do anything else, but I find it very difficult that people see me like this. (w- lung cancer)
				What I have often said to people is that I do not want to be seen as pathetic. And I do notice that people feel sorry for me, especially when I am in the hospital. I am always the youngest and people really looked at you wide-eyed, full of pity. That is difficult. (w- cervical cancer)
		Sadness of loved ones	3.3.2	It is a bit selfish, but it should relieve me. And if they cry very much or show how much it affects them, it just becomes a bigger thing in my head. (w- low-grade glioma)
4. Sense of grief about life	4.1 Grief about the 'old' life	Life 1.0 and life 2.0	4.1.1	I was difficult that also school was taken away from me. And that is not the only thing, because I am also very sporty and then was not able to exercise anymore. Just every time there are those little things you cannot do anymore. (w- lung cancer)
		Grief about the person I used to be	4.1.2	You have to say goodbye to so many things. From the fit [name], the fit mother. I saw myself skiing with the kids, running next to the bike, playing football and going to hockey. Then you have the work [name], the colleague [name], that is also part of my identity. And the energy [name]. They do not really exist anymore. That is very difficult sometimes and it makes me sad to say goodbye to those parts and process this loss. (w- breast cancer)
		The people I left behind	4.1.3	I know how much it will hurt the people I leave behind. It is a kind of guilt 'oh god, what am I doing to these people'. It is not fair. Because we know that when someone dies, people are always left behind and can be very sad about it. The pain that will come when I am death that is what makes me sad and I do not want that. (w- leiomyosarcoma)
	4.2 Anticipatory grief about the life I did not/do not get	Milestones	4.2.1	I had an ideal image of my future and I had adjusted my life for the past 4-5 years in such a way that everything would fall into place. And just on that moment I got cancer. I wanted to go into the consultancy and that includes working weeks of 80-90 hours and I wanted to continue living abroad. Now, I am looking for a part-time position in the consultancy, had to take a step back for my top sport career and was moved in the Netherlands. (m- desmoplastic small round cell tumor)
		Incomplete parenting	4.2.2	The life lessons that you have experienced or learned as a person, that you can no longer pass them on to your child. That he has in the back of his mind: 'yes mom, she always says this and that'. […] And writing it down is different from conveying it yourself and that he knows me as a person. (w- leiomyosarcoma)
		Jealous of peers	4.2.3	I experience a kind of jealousy towards all people who can just do all the things I cannot. And I also experience jealousy towards my friends, because I see them working and starting an internship … and I would like to do that too. And I just find that very difficult. (w- lung cancer)

(Continued)

TABLE 3 Continued

Theme	Sub-theme	Codes	In-text reference	Quotes
5. Loss of control over the future	5.1 Adjusted future perspectives	Missing future guidance	5.1.1	How I organize my life, what I have to take into account is always uncertain. It is like someone is constantly sawing my legs. It stops for a while, then there is a short break and one leg is shorter than the other, but you learn to live and deal with it. And then some saws again, it becomes a bit more skewed or with help a bit straighter again. But you know it never ends. So with a bad scan result, they are sawing again and you do not know how it ends. You did not know the impact from a bad scan result either. (w- breast cancer)
				As long as the treatment works, it is good. But I do not know how long that is and that is really hard for me. So I set goals like school and getting married to stick to them. And if that disappears for me, I do not see the point of either. It is not that I am done with life, but I need something to look forward to. (w- lung cancer)
		Scanxiety	5.1.2	It is just an update if your continue living. That is how it gets into my head. I did not work for a week because I was just completely exhausted from the weeks before the scan. I was ruminating and was exhausted because of the stress. (m- low-grade glioma)
	5.2 Not being able to make (life) choices or plans	No direction for choices or plans	5.2.1	I really need certain goals or things that I can plan. And the fact that you can only look 8-12 weeks ahead, that is not bad for a while but after 4 years it started to frustrate me. After 4 years I was no longer 28 but 32. So quite a lot has changed, but I am standing still or actually go back even further. I can plan a vacation and make small purchases such as a car. But big choices like starting a family is impossible, while our biological clock is ticking and you are confronting with everyone around you who will settle done. (m- melanoma)
				I am now in a position, I am 28 years old and my study is still 2 years. And I really have that frustrating feeling of. okay but what if I only have a year and a half left? Then I spend a year and a half to my education and then I die. And I would find that very frustrating. So I really want to spend the time I have on fun things, on things I really want to do. (w- breast cancer)
		Life rush	5.2.2	I put a little pressure on myself […] A bit of an adult life. I want to do or achieve what my parents have. I think I must have taken everything out. (w- low-grade glioma)
		Unpredictable wellbeing	5.2.3	It is also just hard to plan dates with friends. My friend wants to make an appointment to play a board game or something on Saturday but I had to cancel it. Then he has to keep the next Saturday free and I do not want him to keep his Saturday free if I do not know if I am going to make it. So it makes it more difficult to keep in touch. (m- low-grade glioma)
	5.3 Waiting for growth	Sitting in a waiting room	5.3.1	You are in the waiting room, but you do not know when it is your turn. And there is no indication that you have got 10 minutes left. There is not ten or twenty minute leeway, you do not know what is about to happen. So it is just a matter of waiting. (w- leiomyosarcoma)
		Survivorship guilt	5.3.2	I met a girl during cancer treatment and we became friends. She had a semi- good prognosis and I did not, however, she died and I am still here. I felt guilty regarding her family assuming they did not want to see me since I am still alive and their daughter is not anymore." (w- focus group)
		Curative versus palliative	5.3.3	[…] then at least you are doing it for something. There is a dot somewhere that you live for, like 'Well, I have to do this. I am almost out of strength but I am not giving up because eventually I will be better and then I can live to 100. Then I can start planning my future and get on with my life.' And now you have a lot of trouble and you are almost at that dot at the horizon but that point keeps disappearing, it won't come. You are fighting something you can never win, you know you are going to lose in the end. (w- melanoma)

m, man; w, woman.

It is remarkable that feeling alone is more reported by women than men.

Theme 3. Ongoing confrontation

3.1. It is always there

AYAs report their cancer is 24/7 on their mind; it is part of their daily life (3.1.1). Even when the cancer is stable and people expect that these thoughts move to the background, AYAs express that they always think of the sword of Damocles above their head because of their shortened life expectancy (3.1.2). For the new AYA survivors, the cancer is usually more on the background. However, they experience their fluctuating physical or mental well-being and the hospital appointments as confronting factors, switching the cancer to be on the foreground (3.1.3).

3.2 Own physical and cognitive decline

When denial of the cognitive and physical issues is not possible any longer, AYAs are confronted with their own decline (3.2.1). This is mainly the case by AYAs with low-grade glioma followed by new survivors with an uncertain prognosis. For some AYAs their decline feels like a sacrifice they have to make repeatedly, which impacts every aspect of their life. Others experience anxiety of how this (unknown) cognitive decline ends. In some cases, AYAs are capable to compensate their decline by using mnemonics and other

FIGURE 1
Visual overview of challenges for AYAs with UPCP experiences. The size of the inner circles represents the frequency of the reported challenge, e.g. the bottom two circles are the largest, which means that these challenges were experienced the most.

tricks. This results in the situation that their decline is not always noticed by their environment, with the risk that too much is expected from the AYA (3.2.2). Although, the decline is not always remarkable, AYAs themselves are everyday conscious of how it was before and how it is now.

3.3 Environment

The wish of the AYA to be normal is in contrast with the cancer stigma in the society and the attitudes of the environment towards AYAs with a UPCP. Difficulties with a driving license due to the epilepsy (in low-grade glioma), challenges with buying a house, and not getting a permanent contract are issues contributing to a confrontation that the AYA is no longer participating fully in society. The AYA and the cancer are also often the center of attention of their social network. Concerns of their loved ones are mostly expressed in searching for more contact, taking over tasks and making decisions for the AYA which make the AYA feel more dependent. AYAs are also experiencing that the environment feels sorry for them, which makes the AYA actually feel like a patient (3.3.1). Additionally, the sad emotions of their loved ones make AYAs realize that their situation is indeed serious. This sadness is also too much to handle since they have enough of their own sorrow. Instead of providing relief by sharing thoughts, it provides a conformation of the concerns the AYA has, resulting in protecting themselves by not sharing everything with their loved ones (3.3.2).

Theme 4. Sense of grief about life

4.1 Grief about the old life

AYAs with a UPCP experience grief about their pre-diagnosis life since psychosocial, cognitive, and physical issues

TABLE 4 Differences in experienced challenges between AYA subgroups.

Themes, sub-themes, codes	Less often reported by	More often reported by
1. Feeling inferior to previous self and others	New survivors	
1.1 Feeling not useful		Low-grade glioma survivors
2. Feeling sense of loneliness	Men	
2.3 Nobody gets me	Traditional survivors	
3. Ongoing confrontation	Traditional survivors	
3.1.3 Medical factors		New survivors
3.2.1 I cannot deny anymore	Traditional survivors	
5. Loss of control over the future	-	-
5.1.2 Scanxiety	Traditional survivors	
5.2.1 No direction for choices of plans	Low-grade glioma patients	

cause limitations in all areas of life. Many aspects of their lives have stopped because the cancer or adaptions have been made regarding study, work, and relationships (4.1.1). The whimsical pattern of this uncertain and poor cancer prognosis ensures that people continue to deteriorate and therefore have to give up more and more throughout their lives. This repeated loss experience creates grief about the person they used to be and creates questions about their own identity (4.1.2). Although, this group has no other option than dealing with this grief, most of them cannot accept the changes and have the wish to go back to their old life. Saying goodbye to the old life also includes saying goodbye to their loved ones. Leaving their loved ones behind because of the fact that you will die prematurely is the most painful part for AYAs with a UPCP. It felt like they are going to hurt their loved ones by abandoning them, and not being able to provide comfort makes it emotionally very hard (4.1.3). AYAs experience worries about how their loved ones are coping after their death and some are even afraid that they have not done enough to leave their loved ones behind in the best possible way (e.g. not enough savings). In some cases, these worries resulted in an extreme need of control and others keep people at a distance so they do not have to hurt them either.

4.2 Anticipatory grief for the life I did not/do not get

Many AYAs are working hard to accomplish their future goals. Because of the cancer and its consequences, it is the question whether these goals and other milestones can be achieved including lost parenthood, not being able to buy a house, and giving up your dream job (4.2.1). This asks for a new vision of the future, which often goes hand in hand with ethical dilemmas (e.g. is it ethical to start a family in my situation)?. Furthermore, young parents experience anticipatory grief for missing many milestones from their children, not knowing what kind of person their child will become, and not being able to provide their children with life lessons (4.2.2). Seeing your peers living their life and achieving future goals is experienced as an extra confrontation by AYAs. This causes feelings of jealousy, especially when peers are doing activities AYAs are not capable of anymore (4.2.3). The feeling that they will never achieve the same level as their peers feels unfair and for some AYAs, resulting in the struggle of having trouble to be happy for a peer when achieving a life event.

Theme 5. Loss of control over the future

5.1 Adjusted future perspectives

Majority of the AYAs with a UPCP are forced to adjust their ideas about the future. Instead of most of their peers, AYAs do not think and plan long term and some do not even dare to dream of a future. Most of them are focusing on the current situation and planning a week or a few weeks ahead. Others are planning for a year, hold on to the life expectancy range they received by their specialist, or focus on a goal like graduating or marriage so they have something to live for. Not having a future perspective or even a bit of control and not even knowing what you live for are experienced as the hardest part by these AYAs and asks for an enormous flexibility in their mindsets. This flexibility is being required since scan results and treatment can change their daily life and future direction (5.1.1). Therefore, many AYAs plan their future from scan result to scan result as this determines the rest of their life, which comes with enormous stress and sometimes even impacts their functioning and wellbeing for a period of time (5.1.2). It is remarkable that this stress is not mentioned by many AYAs in the traditional survivor subgroup. Some AYAs experienced their loss of control over the future more easily during the COVID-19 pandemic, since everybody in the Netherlands was forced to adjust their future plans and experienced some uncertainty.

5.2 Not being able to make choices or plans

Most of the new and traditional AYA survivors report that being young and not having a clear future perspective makes it hard to make (life) choices, because a good rationale to base choices on is lacking. “What is a good choice in this situation, what is it worth?” Topics like buying a house, starting a study, quitting your job, and starting a hobby are difficult if nobody can tell you how long you have left to live. The wish to become a parent is an extra ethical discussion (5.2.1). Mainly new and traditional AYA survivors express that they feel out of control and that they are constantly wondering whether they are making the right choices. Several AYAs experience suicidal thoughts, as this will end the uncertainty. Some are struggling with a sense of rush of life; they want to do as much as possible because death can suddenly appear. This creates a lot of pressure, little time for relaxation, and the idea that relaxing is a waste of your time (5.2.2). An extra challenge is the unpredictability these AYAs experience in daily life according to their physical and mental health. Everything they plan is with reservation of their wellbeing, which they have no control of and results often in canceling plans and being disappointed (5.2.3). For some it feels like they are not in the lead of their own lives. AYAs express that during the COVID-19 pandemic, they experience less fear of missing out since everyone was forced to cancel their plans.

5.3 Waiting for growth

AYAs explain that whatever they do the only certainty is that they will eventually die prematurely of cancer. It is hard for them to know that no matter what they do, no matter how tough they are, or how hard they work, the outcome remains the same (5.31). Waiting for growth is for AYAs in the focus group associated with experiencing survivors’ guilt, which implies struggling from simply being alive, especially when many of their peers from the cancer community may not have survived (5.3.2). Sometimes the motivation to get out of bed and undergo

a new treatment is lacking since there is no hope of curation. Except for extra time with hopefully good quality of life and time with their loved ones, a clear and healthy future is missing (5.3.3).

Discussion

With this study we are the first to describe the psychosocial challenges in daily life specifically for the group of AYAs with a UPCP. In collaboration with AYA research partners, we identified four main challenges with an impact on all areas of life: feeling inferior to previous self and others, feeling of being alone, ongoing confrontation, grief about life, and loss of control over the future. Although all of the challenges were identified in the different AYA subgroups (traditional survivors, new survivors, and low-grade glioma survivors), the perceived intensity of the challenges differed slightly between the subgroups. The experienced psychosocial challenges seem not to differ by tumor type or stage of treatment other than those included in the AYA subgroups.

The first challenge, *feeling inferior to previous self and others*, shows that AYAs with a UPCP felt useless since they were not being able to perform their work in the same way as they did before while work was part of their identity and reminded them of their "normal" life. It is remarkable that this was mainly reported by AYAs with a low-grade glioma. A possible explanation could be the dissonance between their physical and cognitive functioning, which makes it even harder to accept the adjustments in work since some of them still had the physical capacity to work but suffered from cognitive dysfunction. Besides this loss of work identity, our results show that AYAs do not see themselves as a good romantic partner since they cannot offer what a healthy peer can offer. In line with previous research, we found that AYAs with UPCP have to deal with many loss experiences, regarding everything they could do in their pre-diagnosis life as well as the person they used to be. They also experienced the loss of their future dreams or lost opportunities to participate in developmental milestones like becoming a parent, which affected their sense of purpose (7, 10, 13).

Related to the identified challenges of *grief about life*, AYAs seems to feel broken, which is an accumulation of three potential causes (1): the differences between the pre-diagnosis and current situations (2), loss of their old life and future life, and (3) the internalized societal thoughts of the AYAs according to their impairment. According to the 'contingent hope theory', periods of feeling broken and experiencing loss may accumulate to a point where AYAs lose orientation to almost every facet of their pre-cancer identity, which is called disorienting grief (7, 10). In our study, disorienting grief was reported by several AYAs. It may seem possible that the other AYAs found themselves in other stages of the theory in which they were focusing on reframing mindsets and daily priorities to balance their experiences of loss or identity reconciliation. Currin-McCulloch et al. portrayed that hope plays an important role in motivating AYAs through disorienting grief toward finding a new balance; however, situational or medical changes can start the process of loss and feeling broken again (7). Additionally, AYAs with a UPCP reported experiencing *ongoing confrontation* since their poor prognosis is always on their mind or medical factors like hospital appointments confronted them on a regular basis. In line with previous research, we found that being conscious about their physical decline and the fact that people treat you differently are additional confronting factors for this patient group (15, 26).

Our results showed that almost nobody truly understood the unique situation of AYAs with a UPCP, which resulted in their *sense of feeling alone*. Studies by Currin-McCulloch and Knox suggest that this might be explained by the mismatch with curative AYAs since they can return to their normal life with future life goals, which was also reported by the AYAs in our study (7, 10, 12). They felt alone in their thoughts about death and dying, felt sidelined, or even took some distance on their own since they wanted to protect their loved ones or do not fit in the old life of their peers anymore. It seems possible that men do not feel the same social needs as women, since these feelings of loneliness were mainly reported by AYA women. Earlier research suggest that men use more problem-focused coping strategies (active efforts to eliminate the stressor) instead of emotion-focused coping (changing the emotional response to stressor), reported information as emotional support, and were possibly reluctant to admit needing emotional support in contrast with women who seek emotional support from numerous sources (27, 28).

All these loss experiences form an extreme burden. On top of that, AYAs with a UPCP are dealing with these losses while not experiencing any *control over their future life* due to medical uncertainties (13). This constantly asks for a flexible attitude to find a new balance in what they are capable of (and what not anymore), who they are, and how their future looks like. Adjusting their future by planning day by day or from scan to scan goes along with not being able to make long-term plans or choices due to their uncertain prognosis. Shiling and colleagues suggest that living with uncertainty is difficult in older melanoma patients, but probably even more difficult in young cancer patients (29), since AYAs have to make many choices about their future life (e.g. should I start a new study, should I start a family) without any hold on or end point to help them making the right choices. The extra difficulty of dealing with advanced cancer in a vulnerable life stage of AYAs is supported by research of Lie et al. suggesting that AYAs have fewer life and coping experiences (26). It is interesting that AYAs with a low-grade glioma did not frequently report issues around a lack of direction for choices or plans due to the uncertain future. It seems that the majority of these patients already have made

choices in life, since just slightly more of these AYAs are in a relationship and are a parent, which may provide some future direction. A relatively longer life expectancy and multiple treatment options may offer another explanation. In some cases, loss of control over the future increases the need to live a meaningful life and rush to accomplish milestones regardless of the time they have left (12). For other AYAs who are not experiencing a rush of life, missing that dot on the horizon sometimes feels like they have nothing to look forward to.

Limitations

While this qualitative study included relatively large numbers of AYAs with a UPCP, the representation of ethnic minority groups was limited, which makes it hard to generalize the conclusions to this entire population. Future research should focus specifically on the challenges of the ethnic minority groups in order to provide culturally appropriate care. Additionally, we were not able to include AYAs below the age of 23 years, and the man/woman ratio was skewed. This slightly limits the generalizability of our results. The data of this study are based on a single interview with each AYA to examine the primary experiences, while it is possible that the challenges experienced by AYAs with a UPCP are changing over time. We aimed to cover the differences in challenges over time by including a heterogeneous group of AYAs with a UPCP with diversity in diagnosis, years since diagnosis, and treatment. Additionally, we asked the respondents if they assume whether their answers would change if they were in a different (disease) phase, to which half of them replied with yes. Longitudinal future research should get a better understanding of the age-specific risk factors that contribute to the development, maintenance, or fluctuation over time of the challenges we identified in this study. This future research may provide an explanation for the differences we found between the three AYA subgroups.

Clinical implications

AYAs with a UPCP get a lot of respect from social media, family, or medical staff on how well they handle their situation, yet they do experience daily challenges. This study highlighted the complexities of AYAs living with a UPCP. These challenges appeared to be specific for AYAs with a UPCP but are not always openly shared by the AYA or are simply not visible for everyone. This calls for healthcare professionals to ask the right questions. We can learn from the example of the Dutch AYA Care Network, which developed an AYA topic list to assist healthcare professionals to examine the typical care needs of AYAs (e.g. fertility, education and work, death, meaning of life) and also provide some interventions to meet their needs (e.g. refer timely to palliative team) (30). Although some topics match with the challenges reported in this study, the current topic list is lacking specific issues or follow-up questions regarding AYAs with a UPCP (e.g. feeling an inferior identity, sense of loneliness – protecting loved ones, loss of control – future plans). Therefore, we recommend adding the identified themes of this study as topics and the sub-themes as follow-up questions to focus on the loss experience of this group. Since AYAs with a UPCP experienced a high level of anxiety for scan results ('scanxiety'), and some of them plan their lives around the returning scan results, it is valuable to look at the practical considerations of the frequency of the scans and the wait time between scans and results in relation to the psychological burden for the patients (31). Remarkably, anxiety regarding scans was less present in the group of traditional survivors, possibly explained by the fact that this group lives by their chemo-scheme instead of scan by scan.

To minimize social isolation and lonely grief, we should focus on peer-support initiatives. Earlier research found that social support was associated with better psychological and existential quality of life and less severe grief in young adults with advanced cancer (32). Trevino and colleagues suggest that providing context to discuss their experiences and meeting their practical needs may be the most effective way of social support (32). However, our study results as well as previous research suggest that AYAs with a UPCP are not able to relate with AYAs with curable cancers (7, 10, 12). It would be beneficial to start peer support groups exclusively for AYAs with a UPCP. Since social support by family and friends cannot be underestimated, AYAs with a UPCP may need assistance balancing the need to be supported by sharing information and withholding of information in relation to their desire to protect their loved ones (15). Additionally, men in this study did not seem to suffer from social isolation; however, we do not know if they are reluctant to seek and accept support. Therefore, gender roles should be taken into account when providing support to make sure than men's needs are not minimized (27). How to provide support to AYA men could be a topic of future research. Furthermore, many respondents commented on how the study interview itself was perceived as helpful, which possibly argues for embedding time to listen and acknowledge the issues of this unique group in daily clinical care programs.

Since the situation and some of the issues this group encounters will never change (e.g. adjustments to work, premature death) and the thoughts and moods AYAs with a UPCP experience may be accurate appraisals of their present circumstances, we have to focus on the things we can change. In AYAs with a UPCP, this means focusing on the relationship with negative thoughts and emotions rather than changing the thoughts and emotions themselves. In this unique patient group, it seems valuable to focus on interventions like Acceptance and Commitment Therapy (ACT), an evidence-based therapy for people learning to deal better with their unpleasant feelings, taking a step away from negative feelings and focusing on the important things in life (33). Although ACT may be beneficial for a range of psychological disorders and some chronic

illnesses and conditions, ACT studies in (advanced) cancer are limited. However, promising pilot data is present (33). Earlier systematic reviews cautiously suggest that ACT may be a beneficial way to improve depressive symptoms, anxiety, psychological distress, psychological flexibility, and aspects of health-related quality of life in adult patients with advanced cancer (34, 35). Future research should focus especially on the coping of AYAs with a UPCP to gain better understanding of how this group can cope with the daily challenges related to their UPCP and how to support them to improve their quality of life. Results show that living longer can also coexist with survivor guilt when identifying with someone who was going through a similar experience and has died. It is an emotional response of guilt connected to a sense of helplessness, powerlessness, and a deep sense of injustice. Since we are used to focus on the positive effects of surviving longer, it is import to be aware of the possibility of survivor guilt in AYAs especially when they are having peer contact (36). Future research should focus on survivor guilt in AYAs more specifically with attention for the risk factors and therapeutic interventions (e.g. cognitive behavioral therapy).

Lastly, this study is far ahead in involving patients as AYA research partners. This collaboration provides more appropriate and relevant research regarding AYAs with a UPCP and contributed to a better translation to suggestions for clinical practice. Furthermore, AYAs were proud to contribute, appreciated the feeling of being seen to be of added value, and received support from the contact moment with other AYA research partners. More details about collaboration with the AYA research partners and its benefits will be presented in a separate article.

Conclusion

This study identified unique challenges of AYAs living with a UPCP, suggesting that the difficulties are particularly associated to their sense of altered identity, their position in the social network, and their future uncertainties. Since specific care for this patient group is net yet embedded in the healthcare system, this study highlights the importance to recognize and acknowledge the unique challenges of AYAs with a UPCP in AYA care programs. Taking into account the differences in perceived challenges between AYA subgroups and gender, it is essential to focus on personalized care. To provide age-specific care, we recommend the implementation of acceptance and commitment therapy and AYA peer support to support AYAs to live well with their UPCP.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of The Netherlands Cancer Institute (IRBd20-205). The patients/participants provided their written informed consent to participate in this study.

Author contributions

OH and WG conceptualized the study and acquired funding. VB, SiF, SuF, NH, MN, AD, WG, and OH developed methodology. VB, MvdB, LD, RL, JT, AC, MK, MB, AdL, and WG contributed to patient recruitment. VB performed interviews and analysis with help of MR. SiF, SuF, NH, MN, AD, WG, and OH were involved in analysis discussions. The original draft was prepared by VB, WG, and OH. All authors reviewed and edited the manuscript. All authors agreed to the published version of the manuscript.

Funding

OH and VB are supported by a grant from the Netherlands Organization for Scientific Research (grant number VIDI198.007).

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.994934/full#supplementary-material

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frontiers | Frontiers in Oncology

TYPE Perspective
PUBLISHED 29 November 2022
DOI 10.3389/fonc.2022.1033993

Check for updates

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 01 September 2022
ACCEPTED 31 October 2022
PUBLISHED 29 November 2022

CITATION
Reschke A, Richards RM, Smith SM, Long AH, Marks LJ, Schultz L, Kamens JL, Aftandilian C, Davis KL, Gruber T and Sakamoto KM (2022) Development of clinical pathways to improve multidisciplinary care of high-risk pediatric oncology patients. *Front. Oncol.* 12:1033993. doi: 10.3389/fonc.2022.1033993

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Development of clinical pathways to improve multidisciplinary care of high-risk pediatric oncology patients

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Clinical pathways are evidence-based tools that have been integrated into many aspects of pediatric hospital medicine and have proven effective at reducing in-hospital complications from a variety of diseases. Adaptation of similar tools for specific, high-risk patient populations in pediatric oncology has been slower, in part due to patient complexities and variations in management strategies. There are few published studies of clinical pathways for pediatric oncology patients. Pediatric patients with a new diagnosis of leukemia or lymphoma often present with one or more "oncologic emergencies" that require urgent intervention and deliberate multidisciplinary care to prevent significant consequences. Here, we present two clinical pathways that have recently been developed using a multidisciplinary approach at a single institution, intended for the care of patients who present with hyperleukocytosis or an anterior mediastinal mass. These clinical care pathways have provided a critical framework for the immediate care of these patients who are often admitted to the pediatric intensive care unit for initial management. The goal of the pathways is to facilitate multidisciplinary collaborations, expedite diagnosis, and streamline timely treatment initiation. Standardizing the care of high-risk pediatric oncology patients will ultimately decrease morbidity and mortality associated with these diseases to increase the potential for excellent outcomes.

KEYWORDS

clinical pathways, pediatric oncology, high-risk patients, hyperleukocytosis, anterior mediastinal mass

Introduction

The use of clinical pathways in pediatric oncology

Clinical pathways are evidence-based tools that feature a multidisciplinary approach to care, detail the steps involved in care, include timeframes or criteria-based progression, and aim to standardize care for a specific clinical problem (1). Their use in oncologic care may limit unwanted variation in clinical approach and improve quality, efficiency, and value of care (2, 3). In adult patients with cancer diagnoses, clinical pathways have been developed primarily for drug treatment regimens (3). There are fewer published pathways for pediatric oncology patients, but those that exist focus on managing complications of cancer treatment such as fever and neutropenia (F&N) (4, 5). Similar standardization and evidence-based coordination of care may be especially helpful for pediatric oncology patients who present with life-threatening complications of their new cancer diagnosis.

A challenge in pediatrics - critically ill oncology patients

While disease-free survival for pediatric patients with a new diagnosis of leukemia or lymphoma has improved dramatically in the last 40 years (6), the outcomes for those who present with a life-threatening disease component remain disproportionately poor. A large retrospective multicenter analysis by Zinter et al. found that while pediatric cancer patients represent 4.2% of pediatric intensive care unit (PICU) admissions, they comprise 11.4% of PICU deaths and have an overall mortality of 6.8% (7). Other publications report an even higher mortality for pediatric cancer patients admitted to the ICU, ranging from 13% to 27.8% (8–10). Unfortunately, despite the overall improvement in oncologic outcomes in recent years, there has not been much progress in the outcomes of critically ill oncology patients. Two particularly high-risk populations include patients presenting with hyperleukocytosis secondary to leukemia, who have a mortality rate of up to 20% in the case of acute myeloid leukemia (11) and patients presenting with an anterior mediastinal mass, who have a 15% risk of respiratory or cardiovascular collapse (12). In these cases, arriving at a diagnosis with the least invasive approach and initiating cancer-directed treatment as quickly as possible are necessary to limit morbidity and mortality. As such, we identified these patient populations as candidates for standardization in management using a clinical pathway to expedite workup and treatment. At our institution, we have developed clinical pathways for the immediate triage and emergent management of two high-risk clinical conditions in pediatric oncology patients: hyperleukocytosis and anterior mediastinal masses.

Hyperleukocytosis pathway

Hyperleukocytosis, an oncologic emergency

Hyperleukocytosis, defined as a white blood cell (WBC) count greater than 100,000/μL, occurs in 10% to 20% of patients with newly diagnosed acute leukemia (11, 13, 14). While hyperleukocytosis is more common in acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) is more likely to cause complications at lower relative WBC counts. The most concerning complication of hyperleukocytosis is leukostasis, a phenomenon that occurs when leukemic blasts occlude the microvasculature, obstructing blood flow to various tissues and leading to end organ damage. Leukemic blasts and endothelial cells interact on the molecular level to release tissue factors and proinflammatory cytokines that worsen tissue damage and lead to coagulopathy (15). Leukostasis may present with a variety of signs and symptoms, including acute kidney injury, oliguria, shortness of breath, hypoxemia, respiratory distress, altered mental status, visual changes, and focal neurologic deficits. Symptomatic leukostasis is a true medical emergency and requires prompt initiation of leukemia-directed therapy to reduce the risk of permanent organ damage or death. Complications related to leukostasis occur more often with AML due to the increased size and poor deformability of myeloblasts.

Hyperleukocytosis pathway highlights

We took the clinical factors described above into account to create a clinical pathway with risk stratification based on the presence or absence of leukostasis, general clinical appearance, and suspected diagnosis from morphology. A mainstay of treatment for all patients with newly diagnosed leukemia – especially those with hyperleukocytosis – is hyperhydration to improve hyperviscosity and reduce the risk of leukostasis. For patients identified to be at low risk (no signs of leukostasis, well-appearing, and with morphology favoring ALL or chronic myeloid leukemia [CML]), leukemia-directed therapy is not initiated until confirmation of diagnosis by flow cytometry. However, symptomatic patients, those who are ill-appearing, or those with morphology favoring AML, acute promyelocytic leukemia (APML), or ambiguous morphology, warrant a more aggressive approach and initiation of immediate empiric therapy.

Of note, there is no definitive evidence demonstrating a mortality benefit for leukapheresis in patients with hyperleukocytosis and hence it is not a part of the treatment algorithm (14, 16). Leukapheresis may be considered in patients with AML with ongoing leukostasis as an adjunct to initiation of chemotherapy (11, 16–18) or patients with CML with evidence of priapism, hearing loss, visual changes, or pulmonary infiltrates (19). Initiation of leukapheresis is often time consuming as it requires placement of specialized central lines and mobilization of multiple services. As a result, our pathway stresses that leukapheresis should not delay initiation of definitive leukemia-directed therapy.

Other complications associated with hyperleukocytosis include tumor lysis syndrome (TLS) with related metabolic derangements and disseminated intravascular coagulation (DIC), which increases the risk of intracranial hemorrhage (ICH). While a separate clinical pathway guides the management of TLS at our institution, the hyperleukocytosis pathway details transfusion parameters, which are modified in patients with DIC and/or ICH. In these patients, the INR goal is lower and the platelet goal is higher to minimize the risk of bleeding.

Another important aspect of the management of hyperleukocytosis is surrounding these transfusion parameters. While these patients are often anemic at presentation, packed red blood cell (PRBC) transfusions in the setting of hyperleukocytosis can increase blood viscosity and result in worsening leukostasis. Therefore, PRBC transfusions should be reserved for patients who are hemodynamically unstable and should be done with extreme caution (20). In contrast, platelets have a minimal contribution to blood viscosity and given the risk of DIC and ICH, platelets should be transfused to maintain a platelet goal >50,000. If there is active concern for DIC or ICH, this threshold should be increased to maintain a plateletgoal >100,000. Figure 1 depicts the most recent hyperleukocytosis pathway currently in use at Lucile Packard Children's Hospital (LPCH), Stanford Children's Health since October 2021.

Hyperleukocytosis pathway development

This pathway was developed by a multidisciplinary group including physicians from both the Division of Pediatric Hematology, Oncology, Stem Cell Transplantation & Regenerative Medicine and the Division of Pediatric Critical Care, incorporating additional input from oncology pharmacists. This pathway underwent multiple iterations to incorporate feedback from members of both divisions. The goal of this pathway is prompt notification of the multidisciplinary team involved in the care of children with hyperleukocytosis to facilitate contingency planning and to minimize morbidity and mortality through the expedited initiation of leukemia-directed treatment.

Anterior mediastinal mass pathway

Anterior mediastinal mass, an oncologic emergency

Anterior mediastinal masses are another well-recognized oncologic emergency due to their precarious location that may result in narrowing or obstruction of the trachea, bronchi, superior vena cava, pulmonary vasculature, great vessels, and heart. Compression of the trachea or bronchi can present with dyspnea, orthopnea, cough, stridor, hoarseness, or chest pain and puts a patient at risk of airway obstruction and respiratory failure. A mass causing compression of the superior vena cava (also known as SVC syndrome) can cause a patient to present with edema of the head, neck, and upper extremities from increased venous pressure and decreased venous return, which can lead to thrombosis, cerebral edema, and hemodynamic instability. Compression of pulmonary arteries can result in decreased pulmonary perfusion and right heart failure, while compression of pulmonary veins can lead to pulmonary edema and decreased cardiac output. Compression of the heart and great vessels can result in decreased cardiac output and put a patient at high risk of cardiovascular collapse (20). In a patient with an anterior mediastinal mass, the degree of cardiorespiratory compromise can be evaluated with computed tomography (CT) and echocardiogram to further inform medical decision making.

Anterior mediastinal mass pathway highlights

Similar to the hyperleukocytosis pathway, we incorporated clinical factors into a risk stratification schema to organize a multidisciplinary approach to determine the ideal diagnostic approach and treatment plan. Patients are considered "high-risk" if there is: 1) tracheal cross-sectional area ≤70% and/or presence of carinal/bronchial compression, 2) significant or near-complete SVC obstruction, 3) pericardial effusion and/or tamponade, 4) pulmonary artery outflow obstruction, or 5) ventricular dysfunction (21). In these cases, general anesthesia poses a significant risk to patients as it can decrease respiratory drive, cause loss of normal negative pressure ventilation, decrease bronchial smooth muscle tone increasing the risk of airway collapse, and cause peripheral vasodilation leading to decreased venous return (20). As such, recommendations include minimizing general anesthesia in favor of using local anesthesia whenever possible, and in conjunction with the oncologist, identifying the least invasive approach to diagnosis. When general anesthesia is necessary, it is important to maintain spontaneous ventilation, avoid muscle relaxants, and ensure the availability of additional equipment and extracorporeal

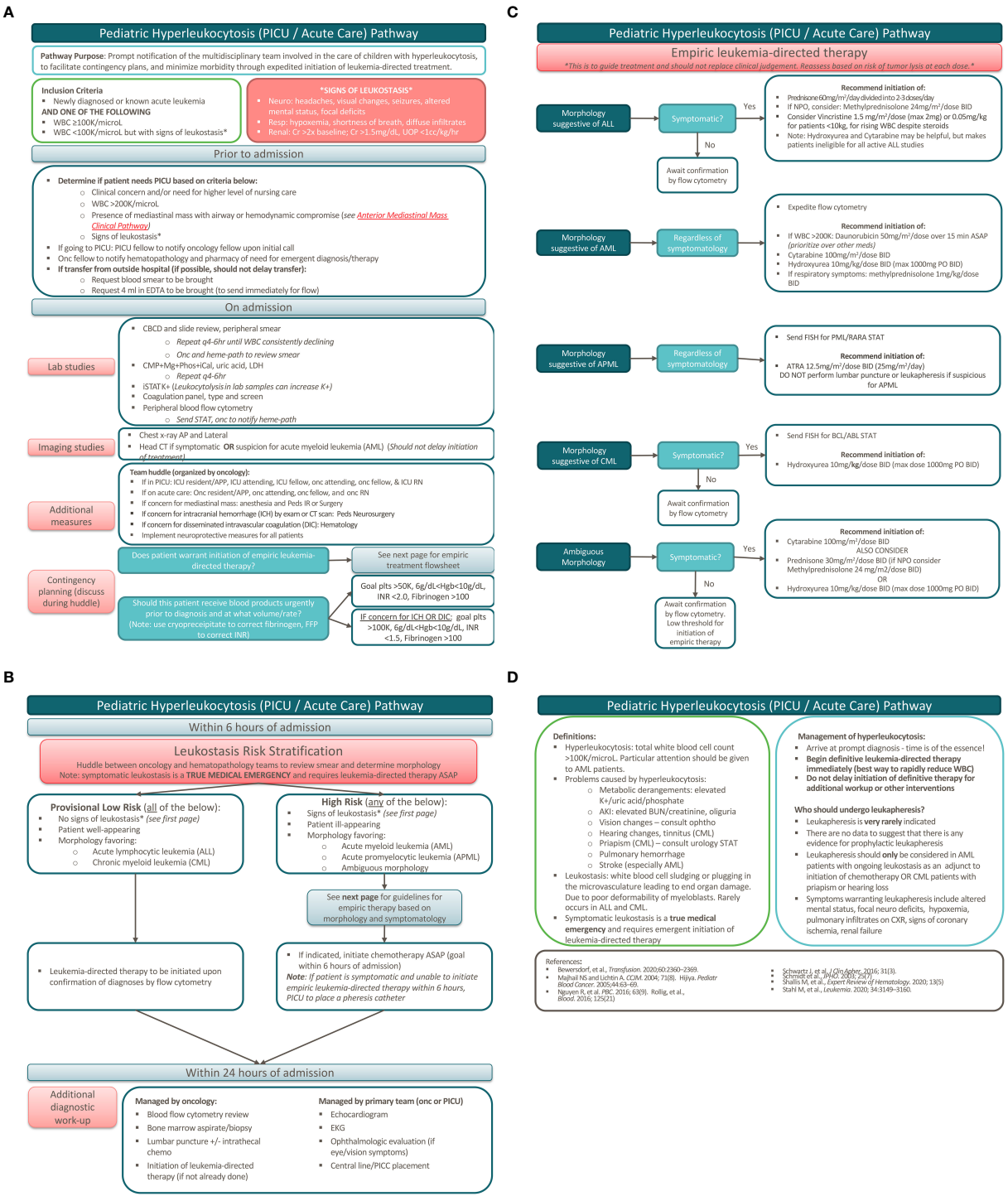

FIGURE 1
Pediatric hyperleukocytosis pathway. **(A)** Pathway overview. **(B)** Patient risk stratification. **(C)** Empiric leukemia-directed therapy. **(D)** Definitions and additional management.

membrane oxygenation (ECMO) in the case of emergency. Even with these precautions, the risk of life-threatening complications from anesthesia in these clinical scenarios has been reported to be as high as 9.4% to 20% (22).

The most common pediatric malignancies associated with anterior mediastinal masses include non-Hodgkin lymphoma (predominantly T-lymphoblastic leukemia/lymphoma) and Hodgkin lymphoma, with a smaller proportion being caused by germ cell tumors, thymomas, and thyroid tumors. Each malignancy is diagnosed differently, so the clinical picture needs to be considered in determining the study with the highest likelihood of yielding a diagnosis (23). While lymphoblastic leukemia or lymphoma can be diagnosed by flow cytometry if there are circulating malignant blasts, bone marrow biopsy can provide a diagnosis in 32% of patients with non-Hodgkin lymphoma and a mediastinal mass when peripheral disease is absent (23). Diagnostic yield of thoracentesis of pleural effusions can be as high as 92% in patients with lymphoblastic lymphoma, but is of limited utility for Hodgkin lymphoma. The most definitive approach regardless of diagnosis is biopsy of the primary site, which can be diagnostic in 86-100% of cases depending on the type of biopsy (23). The site of biopsy is another important consideration. In one study, only 19% of children required biopsy of the primary mass (24). A diagnostic algorithm, such as the one created by Perger et al, guides physicians to start with the least invasive approach and proceed to more invasive approaches if the prior approach was non-diagnostic. While this minimizes the initial procedural risk, it may lead to multiple procedures and a substantial delay in diagnosis. For this reason, we favor a multidisciplinary approach that harnesses each individual's expertise to select a diagnostic strategy that is both high yield and minimally invasive, prioritizing obtaining a diagnostic specimen on the first attempt. Only with prompt diagnosis can definitive disease-directed therapy be initiated swiftly.

The initial mediastinal mass treatment algorithm at our institution was created and published by Hammer in 2004 (25). This pathway has been revised multiple times since then and has incorporated recommendations for anesthetic risk stratification from other studies (21). While 3-level risk stratification strategies have more recently been developed for the management of children with anterior mediastinal masses (22, 26), the approach to intermediate and high-risk patients remains the same, advocating for the avoidance of general anesthesia in both groups when possible. The most recent iteration of the LPCH pathway includes a timeline to expedite a multidisciplinary huddle and determine the diagnostic/treatment approach. Figure 2 depicts the anterior mediastinal mass pathway currently in use at LPCH, Stanford Children's Health since March 2020.

Anterior mediastinal mass pathway development

While the original anterior mediastinal mass pathway at LPCH was developed by Hammer (25), a pediatric intensivist and anesthesiologist, it has since been revised by a multidisciplinary group including physicians from the Divisions of Pediatric Hematology, Oncology, Stem Cell Transplantation & Regenerative Medicine, the Division of Critical Care, the Division of Pediatric Anesthesiology, the Division of Pediatric Surgery, the Division of Pediatric Interventional Radiology, and the Division of Pediatric Radiology. It was presented at the Surgical and Procedural Quality Committee Meeting with members of all subspecialties present and approved for implementation. The goals of this pathway are to: 1) standardize a systematic approach to pediatric patients with an anterior mediastinal mass, 2) enhance communication between various teams, 3) minimize risk to patients and improve patient safety, and 4) expedite diagnosis to initiate treatment as soon as possible.

Pathway implementation

Both pathways are published on the Stanford Children's Health Intranet Clinical Pathways website. They are accessible to all who work at LPCH through a link directly accessible through the electronic health record. Information about the implementation of these pathways was disseminated to all fellows and physicians in the Divisions of Pediatric Hematology, Oncology, Stem Cell Transplantation & Regenerative Medicine and Pediatric Critical Care through joint educational conferences.

Discussion

The risk of mortality for pediatric patients presenting with hyperleukocytosis or a mediastinal mass is well-established. Minimizing time to diagnosis and initiation of treatment is key to improving outcomes. The primary goal of both clinical pathways presented here is to standardize and expedite management for critically ill pediatric oncology patients at diagnosis to decrease morbidity and mortality. Patients who present with hyperleukocytosis or a mediastinal mass are ideal candidates for this type of emergent, focused, multidisciplinary care. These pathways, created by individuals in both the Division of Pediatric Hematology, Oncology, Stem Cell Transplantation & Regenerative Medicine and the Division of Pediatric Critical Care, facilitate collaboration and ensure that all teams are working within a well-defined roadmap on a concrete timeline to achieve these goals. Another important element of these

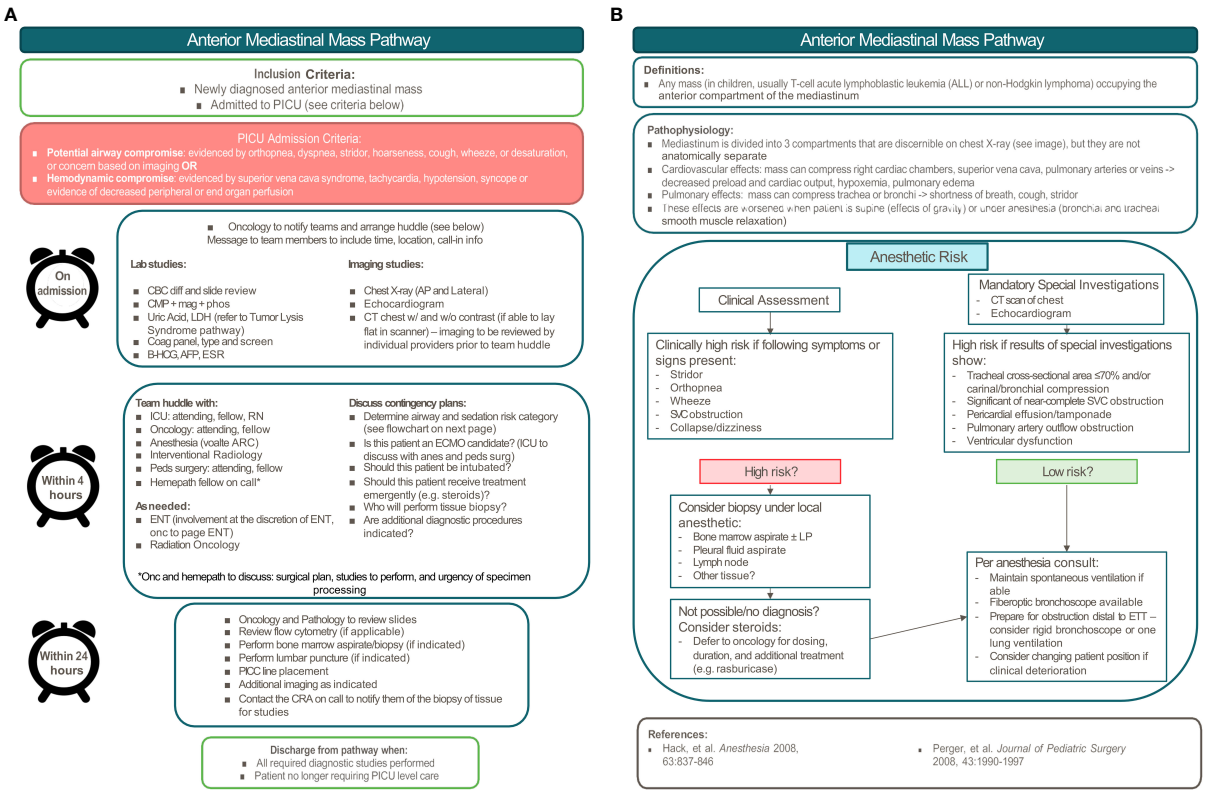

FIGURE 2
Pediatric anterior mediastinal mass pathway. **(A)** Pathway overview. **(B)** Anesthetic risk stratification.

pathways is the multidisciplinary discussion surrounding contingency planning. By anticipating questions that may arise (i.e. transfusion parameters) and discussing them as a team beforehand, we ensure that management decisions limit the patient's risk of an adverse outcome. One especially important point of discussion to occur with patients presenting with an anterior mediastinal mass is the question of ECMO candidacy, and should a patient be deemed high-risk and an ECMO candidate, then plans can be made for the ECMO team to be on standby.

As tissue diagnosis can be obtained from peripheral blood flow cytometry in the vast majority of patients with hyperleukocytosis, this pathway advocates for the initiation of treatment in all high-risk patients without performing either a bone marrow evaluation or a lumbar puncture unless it is absolutely necessary. Awaiting completion of these procedures can lengthen the time to initiation of definitive treatment, further increasing a patient's risk of leukostasis and end organ damage. In contrast, for low-risk patients, we often obtain both the bone marrow aspirate/biopsy and lumbar puncture prior to initiation of treatment as is standard of care.

The focus on obtaining diagnostic tissue prior to initiation of treatment is an important paradigm for treating patients who present with a mediastinal mass. Empiric therapy can result in a delay in diagnosis or rarely, inability to obtain a diagnosis, as these tumors are often very steroid and chemo-responsive. In some cases, empiric treatment may upstage a patient due to pre-treatment status, which could result in more aggressive therapy and thus may increase the risk of treatment-related adverse effects. Empiric therapy before obtaining a diagnostic specimen should be reserved as a lifesaving measure in patients in or at high-risk of respiratory or cardiovascular failure in whom diagnostic tissue cannot be safely obtained.

A large focus of many pediatric oncology practices around the country is to provide patients with the opportunity to enroll in therapeutic clinical trials as data has demonstrated improved outcomes when enrolled (27). In developing the treatment algorithm for empiric leukemia-directed therapy in patients with hyperleukocytosis, careful consideration was given to choosing chemotherapeutic agents that would be effective and would not preclude a patient's ability to enroll in a clinical trial whenever possible.

While multidisciplinary approaches to the management of pediatric patients with anterior mediastinal masses have been described in the literature (24) and anterior mediastinal mass pathways have been created and implemented at other institutions (21, 23, 28), this pathway provides more comprehensive and directed guidance including a suggested timeline, contingency planning, and risk stratification. This is also the first published pediatric hyperleukocytosis clinical pathway and likewise provides a thorough and comprehensive approach to the management of these patients. This pathway also highlights a novel approach to these patients wherein the urgency of initiation of treatment is determined by presence of or estimated risk of leukostasis. Initiation of empiric therapy is often essential for high risk patients, though there are no current consensus guidelines for which agents (or the specific dosing) to select. During the creation of this pathway, we developed recommendations for empiric leukemia-directed therapy based on expertise and consensus within the faculty and pharmacists of the LPCH Division of Hematology, Oncology, Stem Cell Transplantation & Regenerative Medicine.

The development and successful implementation of hyperleukocytosis and anterior mediastinal mass pathways demonstrates the feasibility of standardizing the approach to these patients. While some aspects of these pathways may be controversial and may not be achievable at all institutions, we hope pediatric institutions can use these as a framework to develop their own clinical pathways to further improve outcomes for these vulnerable patient populations.

Future Directions

Through the standardization and expedition of management for these uniquely at-risk pediatric oncology patients, we hope to improve patient outcomes. An ongoing effort at our institution is to provide practical implementation tools including the development of hyperleukocytosis and anterior mediastinal mass order sets in Epic that are directly linked to this pathway. In addition, we are currently developing hyperleukocytosis chemotherapy roadmaps and electronic treatment plans to expedite delivery of chemotherapy to the patient to facilitate treatment. We anticipate that these efforts will further streamline the care of these patients and improve time to treatment.

The anterior mediastinal mass and hyperleukocytosis pathways were launched in March 2020 and October 2021, respectively. After both pathways have been live for three years, we will conduct a single institution retrospective review to measure time to administration of chemotherapy and/or steroids prior to and after the implementation of each of these pathways. Secondary outcomes will include morbidity, mortality, and PICU length of stay. We anticipate improvement of outcomes with full implementation of these pathways, and we envision collaborative efforts with existing pediatric oncology-PICU networks to continually improve standards of care for critically ill pediatric oncology patients.

Data availability statement

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

Author contributions

All of the authors contributed to writing, editing, and organization of the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors thank Michael Link, MD, Kate Steffen, MD, Felice Su, MD, Norman Lacayo, MD, Sean Green, PharmD, Shellie Josephs, MD, Kimberly Hazard, MD, Dita Gratzinger, MD, James Dunn, MD, Bill Chiu, MD, Gregory Hammer, MD, and James Fehr, MD at Lucile Packard Children's Hospital, Stanford for their contributions to the development of these pathways.

Conflict of interest

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

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TYPE Original Research
PUBLISHED 08 December 2022
DOI 10.3389/fonc.2022.1016343

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 11 August 2022
ACCEPTED 16 November 2022
PUBLISHED 08 December 2022

CITATION
Petrilli LL, Fuoco C, Palma A, Pasquini L, Pericoli G, Grabovska Y, Mackay A, Rossi S, Carcaboso AM, Carai A, Mastronuzzi A, Jones C, Cesareni G, Locatelli F and Vinci M (2022) Inter and intra-tumor heterogeneity of paediatric type diffuse high-grade gliomas revealed by single-cell mass cytometry. *Front. Oncol.* 12:1016343. doi: 10.3389/fonc.2022.1016343

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Inter and intra-tumor heterogeneity of paediatric type diffuse high-grade gliomas revealed by single-cell mass cytometry

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Paediatric-type diffuse high-grade gliomas (PDHGG) are aggressive tumors affecting children and young adults, with no effective treatment. These highly heterogeneous malignancies arise in different sites of the Central Nervous System (CNS), carrying distinctive molecular alterations and clinical outcomes (inter-tumor heterogeneity). Moreover, deep cellular and molecular profiling studies highlighted the coexistence of genetically and phenotypically different subpopulations within the same tumor mass (intra-tumor heterogeneity). Despite the recent advances made in the field, the marked heterogeneity of PDHGGs still impedes the development of effective targeted therapies and the identification of suitable biomarkers. In order to fill the existing gap, we used mass cytometry to dissect PDHGG inter- and intra-heterogeneity. This is one of the most advanced technologies of the "-omics" era that, using antibodies conjugated to heavy metals, allows the simultaneous measurement of more than 40 markers at single-cell level. To this end, we analyzed eight PDHGG patient-derived cell lines from different locational and molecular subgroups. By using a panel of 15 antibodies, directly conjugated to metals or specifically customized to detect important histone variants, significant differences were

highlighted in the expression of the considered antigens. The single-cell multiparametric approach realized has deepened our understanding of PDHGG, confirming a high degree of intra- and inter-tumoral heterogeneity and identifying some antigens that could represent useful biomarkers for the specific PDHGG locational or molecular subgroups.

KEYWORDS

paediatric-type diffuse high-grade gliomas (PDHGG), DMG, GBM, DIPG, single-cell, mass cytometry, heterogeneity

1 Introduction

Paediatric-type diffuse high-grade gliomas (PDHGG) are still virtually uncurable brain malignancies that affect children and young adults (1–6). Despite the recent efforts, the scientific advances made so far have not yet been translated into better patient outcome and the overall survival for most PDHGG patients is less than 15 months. The standard treatment still consists of surgical resection (whenever possible), radiation and chemotherapy (6–10). PDHGG can arise anywhere within the Central Nervous System (CNS) but about half of the lesions occur in midline locations. Since they are highly diffuse and infiltrative at diagnosis, they are impossible to resect (6, 11). In particular, the lesions affecting the pons are associated with the worst prognosis (1) and are not amenable to surgery given the critical role of the pons in controlling all vital functions (9). A major challenge for the implementation of effective therapies is the highly heterogeneous nature of PDHGG, known to drive processes such as cell proliferation, survival, invasion and migration as well as resistance to therapy. From this point of view, the PDHGG heterogeneity is a key obstacle hampering the successful implementation of treatment options and the improvement of patient survival rate (12). Recent molecular profiling and meta-analysis studies have shed light on the different PDHGG molecular subgroups and their clinico-pathological features, i.e. typical locations, histopathological features, age of onset and clinical outcome (6, 13–15). In the more recent classification of CNS tumors elaborated by the World Health Organization (WHO), the designation of the specific tumor entities reflects the "diffuse" nature of PDHGG, also discriminating them according to the location and the association with unique molecular alterations (4). The diffuse midline glioma H3K27-altered (DMG-H3K27) type includes all the tumors of the midline structures of the CNS, harboring the known K27M amino acid substitution on the H3 histone variants of *H3F3A, HIST1H3B*, *HIST1H3C* and *HIST2H3C* genes. Somatic mutations of *ACVR1* (16–19) are found almost exclusively in the pontine lesions, concomitantly to H3.1K27M mutation. Other alterations in genes such as *EGFR* (mutations or amplification) in H3K27M mutants or *EZHIP* (overexpression) in H3 wild-type midline tumors have also been identified and contributed to the most recent definition of the DMG-H3K27 tumor subtype. The hemispheric lesions are largely divided into diffuse paediatric-type high-grade glioma H3 wild-type and IDH1 wild-type (DPHGG-WT) and the diffuse hemispheric glioma H3G34-mutant (DHG-H3G34) associated with the *H3F3A* G34R/V driver mutations. In addition, the infant-type hemispheric gliomas (IHG) (4, 15) identify a group of tumors specifically affecting the infant/young child population (0-4 years old) and characterized by fusion genes involving *ALK, ROS1, NTRK1/2/3, MET*. Besides the inter-tumor heterogeneity, it has been shown that PDHGGs are characterized by profound intra-tumor heterogeneity, at genomic and phenotypic level. By using whole genome and whole exome sequencing, intra-tumor heterogeneity was demonstrated in biopsy, resection and autopsy samples, including specimens collected from multiregion and longitudinal sampling (12, 20–22). Taking advantage of PDHGG patient-derived cell lines, we have demonstrated that genomic and phenotypic heterogeneity are linked and that these tumors included distinct and heterogeneous subpopulations which interact in a functional network that confers a more aggressive phenotype and resistance to treatment, thus narrowing even further the therapeutic options for these diseases (12). In light of these considerations, there is an urgent need to fully characterize PDHGG tumor heterogeneity. The understanding of the specific cell populations and their cellular states contributing to tumor behavior, progression and response to therapy, may path the way toward future therapeutic strategies for patients with PDHGG. Such a challenging goal can be realized by exploiting the potential of a single-cell approach instead of relying on bulk tissue analysis as performed by most studies that failed in the attempt of adequately capturing tumor heterogeneity. With single-cell RNA sequencing approaches, we have started to gain more insights on the cellular lineage of glioma cells and on their plasticity through four main cellular states (neural-progenitor-like, oligodendrocyte-progenitor-like, astrocyte-like, and mesenchymal-like) dictated by the genetic make up and by

the tumor microenvironment (23–25). Here we employ single-cell mass cytometry (cytometry by time-of-flight, CyTOF) (26, 27), a powerful tool that allows to simultaneously study the expression of multiple proteins (over than 40 targets) at single-cell level by means of antibodies linked to rare heavy metal isotopes. Compared to other single-cell modalities, this technology does not restrict the investigation at one level, but enables to define multiple cellular features such as protein expression level as well as post-translational modifications within the same experiment, providing a high-throughput marker quantification with single-cell resolution. We take advantage of single-cell mass cytometry to profile, for the first time, a panel of eight patient-derived cell lines from different locational and molecular PDHGG subgroups, to dissect their cellular heterogeneity at the protein level. The antibody panel adopted for the analysis included 15 markers, specifically set to recognize antigens expressed on the surface and in the intracellular compartments of brain and PDHGG tumor cells through the use of antibodies directly conjugated to metals or, in part, specifically customized to detect the unique histone variants. Our data revealed great phenotypic heterogeneity among the analyzed PDHGG cell lines and highlighted that the degree of plasticity, as well as the clusters of cells populating each cell line, differ from tumor to tumor. Moreover, it also allowed to identify key antigens specifically associated with particular PDHGG subgroups that were further investigated through RNA-seq and immunohistochemistry on a more extended panel of tumor samples.

2 Material and methods

2.1 Cell lines and culture conditions

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethical Committee of the Bambino Gesù Children's Hospital (Ethical Committee Approvals N°1680/2018 and 2297/2020). Informed consent was obtained from all subjects involved in the study. PDHGG patient-derived cell lines were generated immediately after sample collection or from live cryopreserved tissue. Tumor tissue samples were obtained from seven patients at the "Ospedale Pediatrico Bambino Gesù (OPBG)" in Rome (Italy) and one patient at the "Hospital Sant Joan de Déu (HSJD)" in Barcelona (Spain). Cell cultures were established as previously described (28). Briefly, tumor samples were finely minced with a scalpel under a sterile hood. Homogenized tissue was gently enzymatically digested for 20 minutes at 37°C in a solution containing Liberase TL (Roche) and 1 U/mL DNase I (Thermofisher Scientific) diluted in 1X Phosphate Buffer Solution (PBS) (PanBiotech). The reaction was inactivated with Tumor Stem Medium (TSM) consisting of 1:1 Neurobasal-A Medium (Gibco) and DMEM/F-12 (Gibco) supplemented with 10 mM HEPES Buffer Solution (1 M, Gibco), 1X Non-Essential Amino Acid (100X, Gibco), 1X GlutaMAX-I Supplement (100X, Gibco), 1 nM Sodium Pyruvate Solution (100 nM, Gibco) and 1X Antibiotic-Antimycotic (100X, Gibco) and cell suspension was centrifuged at 1300 rpm for 5 minutes. Red blood cell lysis was performed by incubating cell suspension in a solution consisting of 1:1 ACK Lysis Buffer (Gibco) and TSM for 1 minute at room temperature (RT). The reaction was inactivated with TSM and sample was filtered with 70 µm cell strainers (Miltenyi Biotec) prior to being centrifuged for 5 minutes at RT. To initiate and expand primary stem-like cultures, minced tissue pellet was resuspended in TSM with the following supplements: 1X B-27 Supplement (50X, Gibco), 20 ng/mL human bFGF, 20 ng/mL human EGF, 10 ng/mL human PDGF-AA, 10 ng/mL human PDGF-BB (Peprotech) and 2 ng/mL heparin (Stem Cell Technologies) (TSM^+). Live-cryopreserved tissue was gently but quickly thawed at 37°C, transferred into 10 ml of TSM^+ and centrifuged at 1300 rpm for 5 min. The supernatant was gently removed and tissue pellet was resuspended in 1 ml of TSM^+ and mechanically dissociated. For this study, PDHGG patient-derived cultures were initiated and expanded as adherent on laminin (10 µg/mL, Merck), on precoated tissue culture flasks. Cell cultures were routinely authenticity verified, using Short Tandem Repeat (STR) DNA fingerprinting service provided by Eurofins Genomics (Table S1) and tested for mycoplasma.

2.2 DNA extraction and sanger sequencing

DNA extraction and sanger sequencing was performed as previously described (28).

2.3 Immunofluorescence assay

For the immunofluorescence assay, cells were seeded onto laminin (10 µg/mL, Merck) precoated chamber slides. Once cells were subconfluent, the medium was removed and PDHGG adherent cells were washed with 1X PBS and fixed with 4% paraformaldehyde (PFA) for 10 minutes at RT. Cells were washed twice with 1X PBS, permeabilized with 0.5% Triton X-100 in 1X PBS for 10 minutes at RT and non-specific bindings were blocked with 10% Normal Goat Serum (NGS) in 1X PBS for 1 hour at RT. Incubation was performed by diluting metal-tagged primary antibodies in a solution containing 1% Bovine Serum Albumin (BSA) and 2% NGS in 1X PBS (IFF). H3K27M-175Lu (Abcam #ab190631, RRID : AB_2860570 metal conjugated antibody, 1:100) incubation was performed for 1 hour RT while H3.3G34R-170Er (RevMab, #31-1120-00, RRID : AB_2716433 metal conjugated antibody, 1:100) incubation was performed for 20 minutes at 37°C. Cells were then washed twice

and incubated with Goat anti-Rabbit secondary antibody (Alexa Fluor 488, ThermoFisher) diluted in IFF for 1 hour at RT. Nuclei were counterstained with 1 mg/ml Hoechst33342 (Invitrogen) for 5 min at RT. Samples were acquired using LEICA fluorescence microscopy (DMi8).

2.4 Mass cytometry workflow

2.4.1 Preparation of single-cell suspensions for CyTOF

For single-cell mass cytometry experiments, 3 x 10^6 cells of each sample were used. Once removed medium and washed twice with 1X PBS w/o Calcium and Magnesium (Euroclone), adherent cells were incubated with Accutase (Carlo Erba) for 5 minutes. Detached cells were resuspended in TSM$^+$ and centrifuged at 1300 rpm for 5 minutes. Viability staining was performed by incubating cell suspensions in Rh-103 (Fluidigm), diluted 1:500 in TSM$^+$ for 15 minutes at 37°C. Reaction was inactivated with TSM$^+$ and cells were centrifuged for 5 minutes at 1300 rpm at RT.

2.4.2 Cell barcoding

To minimize inter-sample antibody staining variation a palladium-based barcoding approach on fixed cells was applied. Cells were fixed with 1 mL of Fix I Buffer (Fluidigm) and incubated for 10 minutes at RT. The fixation was quenched by adding the Barcode Perm Buffer (Fluidigm) and the different samples were centrifuged at 800 g for 10 minutes. Samples were individually barcoded by incubating cell pellets with the appropriate combination of Palladium isotopes from the Cell-ID™ 20-Plex Pd Barcoding Plate (Fluidigm) in Barcode Perm Buffer for 30 minutes at RT. The staining was quenched with MaxPar Cell Staining Buffer (Fluidigm) and cells were centrifuged at 800 g for 10 minutes.

2.4.3 Antibodies for mass cytometry and antibody staining

Most of the metal-tagged antibodies employed in the study were purchased from Fluidigm. In order to comply the absence of available conjugated antibodies targeting the specific mutated histone proteins, histone primary antibodies were bought and conjugated with metals. H3K27M antibody (Abcam #ab190631, RRID : AB_2860570) was purchased from the vendor and Magne® Protein G (Promega) Purification kit was used according to manufacturer's instructions for antibody purification prior to in-house conjugation with metal. Carrier-free antibody was then conjugated using the MaxPar X8 antibody-labeling kit (Fluidigm) according to manufacturer's instructions. The yield of the antibody retrieved after the conjugation step was assessed by a plate reader (Synergy H1, BioTek, RRID : SCR_019748) and antibody was stored at 4°C at the concentration of 0.5 mg/ml in stabilizing solution (Candor Biosciences) supplemented with 0.05% sodium azide. H3.3G34R antibody (RevMab, #31-1120-00, RRID : AB_2716433) was purchased from the vendor in a functional grade formulation and conjugated with metal tag (170Er) by taking advantage of the MaxPar Antibody Conjugation Service (Fluidigm). For the antibody staining with metal tagged antibodies, samples were pooled into one tube and the surface antibody staining protocol was performed according to manufacturers' instructions. After incubation for 30 min at RT, cells were washed twice with MaxPar Cell Staining Buffer (Fluidigm) and permeabilized with 100% ice cold methanol for 10 minutes on ice. Upon membrane permeabilization, cells were washed twice with MaxPar Cell Staining Buffer (Fluidigm) and incubated with antibodies against intracellular antigens for 30 minutes at RT according to manufacturers' instructions. The full list of antibodies is detailed in Table 1. After intracellular antibody staining, cells were washed twice with MaxPar Cell Staining Buffer and incubated overnight at 4°C in the intercalator Iridium (191Ir-193Ir) (Fluidigm) according to manufacturer's instructions.

2.4.4 Data acquisition

Before acquisition, cell suspension was washed once with MaxPar Cell Staining Buffer and twice with MaxPar Water and filtered through 30 µm filter-cap FACS tube. Cells were then resuspended at 2.5 x 10^5 cells/mL in MaxPar Water containing 10% of EQ™ Four Element Calibration Beads (Fluidigm) and acquired on a CyTOF1 mass cytometer system (Fluidigm).

2.5 RNA sequencing analysis

RNAseq dataset are from Mackay et al., 2017, Mackay et al., 2018, Carvalho et al., 2020 and Izquierdo et al., 2021 (6, 10, 29, 30). Data was aligned with STAR to ensembl hg37, counted using HTSeq and normalized with rlog transformation in DESEq2. Data for cell cultures were from a total of 68 individual patients (H3.1K27M n=7; H3.3G34RV n=5; H3.3K27M n=33 and WT n=23) while data for tumors were from 133 individual patients (H3.1K27M n=5; H3.3G34R n=10; H3.3K27M n=52; WT n=66).

2.6 Immunohistochemistry and image analysis for GFAP expression on tumor tissue slide

Immunohistochemistry was carried out on formalin-fixed paraffin-embedded (FFPE) sections using an automated

TABLE 1 Summary of the 15 antibodies used for the mass cytometry analysis.

Antibody	Tag	Company	Product Identifier #	RRID
Anti-Human CD31	145Nd	Fluidigm	3145004B	AB_2737262
Anti-Human CD34	149Sm	Fluidigm	3149013B	AB_2756285
Anti-Human CD63	150Nd	Fluidigm	3150021B	
Anti-Human CD36	152Sm	Fluidigm	3152007B	AB_2802106
Anti-Human CD29	156Gd	Fluidigm	3156007B	
Anti-Human CD90	159Tb	Fluidigm	3159007B	AB_2893063
Anti-Human CD140α	160Gd	Fluidigm	3160007A	
Anti-Human CD49c	161Dy	Fluidigm	3161016B	
Anti-Human CD56	163Dy	Fluidigm	3163007B	
Anti-Human CD61	165Ho	Fluidigm	3165010B	
Anti-Cross GFAP	143Nd	Fluidigm	3143022B	
Anti-Human Nestin	151Eu	Fluidigm	3151013A	
Anti-Human Musashi-1	155Gd	Fluidigm	3155013B	
Anti-Human H3.3G34R*	170Er	RevMab	31-1120-00	AB_2716433
Anti-Human H3K27M*	175Lu	Abcam	ab190631	AB_2860570

The panel shows the antibody target, the metal isotope tag, the manufacturer company and the relative product identifier number (#). (*) Asterisks denote antibodies that were custom conjugated using either the Antibody Conjugation Service (Anti-Human H3.3G34R) or MaxPar Metal Conjugation Kit (Anti-Human H3K27M).

immunostainer (Dako Omnis). A primary antibody directed against GFAP (polyclonal, prediluited, high pH, DAKO) was applied. GFAP stained tissue slices were acquired using the Nanozoomer (Hamamatsu, RRID : SCR_022537) instrument. Slides were scanned at 40X and images were saved into.ndpi format and viewed using the NDPIv2 software (Hamamatsu). 3 random images at 20X magnification were exported from 11 PDHGG patient tissues (n=5 for H3.1 K27M; n=6 for H3.3 K27M) as.TIFF file and analyzed using the ImageJ software (RRID : SCR_003070, http://imagej.nih.gov/ij/) as described in Negm et al. (31). The mean intensity feature evaluated for each image was normalized over the number of manually counted nuclei for each image.

2.7 Quantification and statistical analysis

2.7.1 Mass cytometry data normalization and gating

After the acquisition, raw data was bead-normalized using CyTOF software and cells were assigned back to their initial samples (debarcoded) by using the commercially available debarcoder software (Fluidigm). Normalized data were then uploaded onto the Cytobank (RRID : SCR_014043) environment to perform initial gating strategies (Figure 1). Briefly, cells were manually gated from debris on the basis of DNA content monitored by the incorporation of the Iridium (Ir) intercalator. Doublets were then excluded according to the event length parameter and single live cells were finally manually gated by using the Rhodium (Rh103) intercalator signal.

2.7.2 Data visualization, analysis and accessibility

For Figures 2, 3, manually gated singlet ($191Ir^+$ $193Ir^+$), viable ($103Rh^-$) cell events were imported in Cytobank and t-distributed stochastic neighbor embedding (t-SNE) analysis was performed launching the viSNE (32) implementation in Cytobank. A proportional event sampling was selected and CD markers were chosen for clustering. The heatmap in Figure 4A was made with R heatmap package while marker expression in Figures 4B, C and Figure 5 were derived from data processed with Cytofkit library in R environment (33), setting the following parameters: merge method: "all", transformation method: "cytofAsinh, cluster method: "Rphenograph" with k=20, perplexity: 30, iterations: 1000, seed: 1982. UMAP analysis shown in Figures 6A, 7 and Figure S3 was made using CATALYST library (RRID : SCR_017127) in R environment (34) by subsampling $1x10^4$ cells. For Figure 7 all markers were selected for clustering and downstream analyses with the exception of H3K37M and H3.3G34R. The multidimensional scaling (MDS) illustrated in Figure 6B was performed on the.fcs files by using the R CATALYST (34). All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad software Inc., San Diego, CA, USA, RRID : SCR_000306). Scatter dot plots show mean values ± SEM. Box plots show minimum to maximum values. Statistical significance was evaluated by the *t* test. Figures were prepared in Illustrator (Adobe, RRID : SCR_010279). Mass cytometry data have been deposited in the ZENODO open repository (https://zenodo.org/), developed under the European OpenAIRE program and operated by CERN (DOI: https://doi.org/10.5281/zenodo.7310971).

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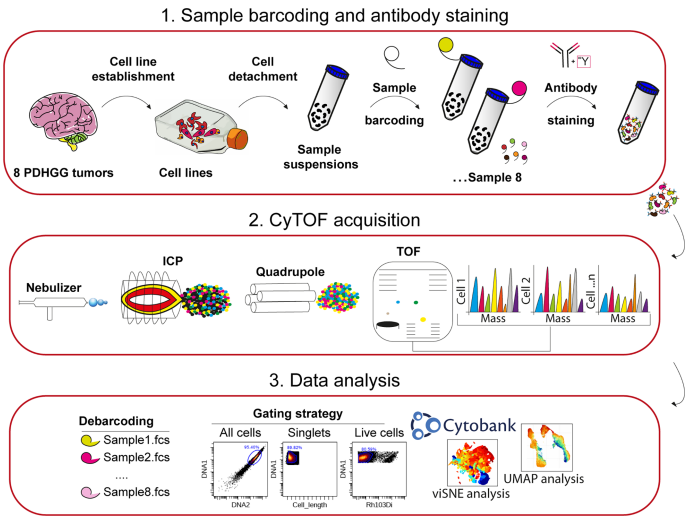

FIGURE 1
Experimental workflow adopted for single-cell mass cytometry analysis. 1. Eight PDHGG primary cell lines were established upon dissociation from tumor patients. After short expansion in stem-cell culture conditions, cells were detached for mass cytometry analysis. The different cell suspensions were barcoded with unique combinations of heavy metal tags and pooled together prior to staining with the selected metal-tag antibodies. 2. The cells were nebulized into a spray of single-cell droplets as they were introduced into the mass cytometer and atomized and ionized by the plasma (ICP). The resulting ion cloud was selected by the quadrupole for heavier reporter masses (>100 Da) which were profiled and quantified on their Time-Of-Flight (TOF). 3. Data were converted to.fcs file and further debarcoded and analyzed in the Cytobank environment, by employing the viSNE tool, and UMAP algorithm.

3 Results

3.1 Development and validation of a CyTOF antibody panel for PDHGG

The antibody panel was designed to include 15 antibodies, selected to profile markers relevant to primary PDHGG patient-derived cells (Tables 1, 2). In particular, the panel included extracellular and intracellular antigens expressed on normal brain and tumor cells (*e.g.* GFAP, CD140α, CD90), stem cells and glioma cancer stem cells (*e.g.* Nestin, Musashi-1, CD34). Integrins and adhesion molecules, particularly relevant for the highly infiltrative nature of PDHGG, were also included (*e.g.* CD29, CD61, CD49c, CD56). Moreover, we included two markers that uniquely identify the two mutations H3.3G34R and H3K27M, associated respectively with DHG-H3G34 and H3DMG-K27M mutant cells; these antibodies were custom conjugated and validated (Figure S2).

3.2 CyTOF profiling of PDHGG patient-derived cell lines

To gain insight into PDHGG tumor heterogeneity through a single-cell mass cytometry approach, eight patient-derived cell lines, were established from fresh tumor tissue specimens collected through biopsy and resection procedures (Table 3) and grown adherent on laminin. The cell lines were derived from hemispheric and pontine tumors and included the main molecular subgroups: two PDHGG-WT, two DHG-H3G34 and four DMG-H3K27, of which there were two H3.3K27M and two H3.1K27M.

For mass cytometry analysis, primary cells were detached and the single-cell suspensions were barcoded, pooled together in a unique tube and stained with the panel of 15 metal-tag antibodies (Table 1) against surface and intracellular antigens (Figure 1). The stained single-cell suspension was analyzed with a CyTOF1 mass cytometer instrument and, after signal debarcoding, single-cell data were analyzed by applying the t-Distributed Stochastic Neighbor Embedding (t-SNE), the algorithm implemented in Cytobank, and UMAP (Figure 1) (54).

3.3 Multiparametric profiling of PDHGG patient-derived cell lines

To visualize the heterogeneity of PDHGG patient-derived cell lines, we generated two-dimensional graphs using tSNE algorithm in Cytobank which is used to analyze the protein expression at single-cell level. All the considered patient-derived cell lines were negative for the expression of angiogenic cell markers such as CD31 and CD36, which have been previously found also on glioma cancer stem cells (35, 38, 39, 46) (Figure 2). For other markers tested in the study, such as the cell-adhesion

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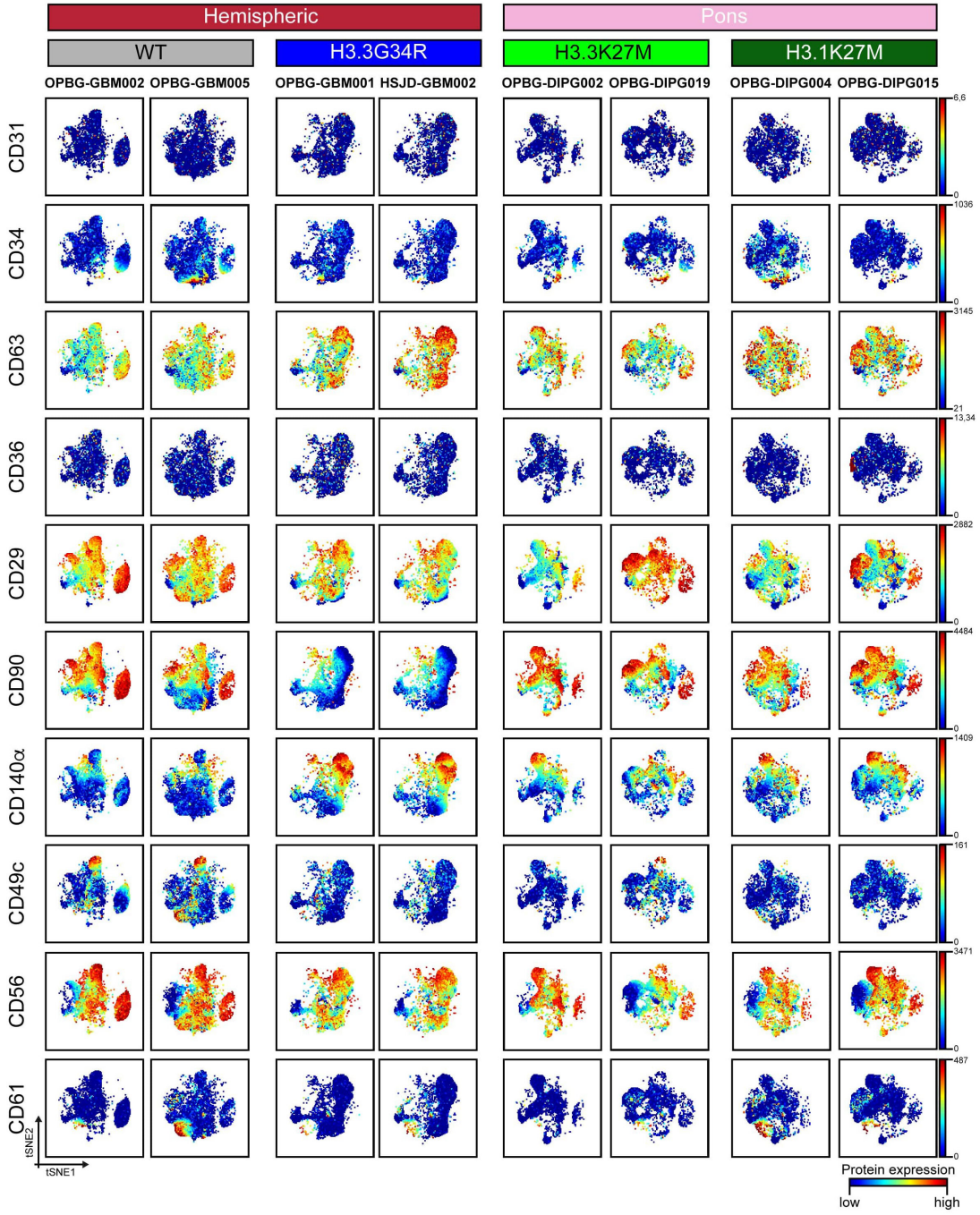

FIGURE 2
CyTOF single-cell analysis of surface antigens in PDHGG patient-derived cell lines. t-SNE maps showing the expression of 10 surface markers (CD31, CD34, CD63, CD36, CD29, CD90, CD140a, CD49c, CD56 and CD61) in each of the eight different PDHGG patient-derived cell lines analyzed through mass cytometry technique. The color gradient refers to the intensity of the expression of the considered marker, in a blue to red scale indicating low and high intensity respectively.

molecule CD56, the integrin β-1 CD29 and its activator CD63, the tyrosine-kinase CD140α and the cell membrane molecule CD90, a variable degree of antigen expression was observed in each individual cell line. In fact, when individually considering each patient-derived cell line, the expression of the abovementioned markers was highly heterogeneous, ranging, for each marker, from a low expression level, indicated in blue, to a high expression level shown in red (Figure 2). This intra-tumor

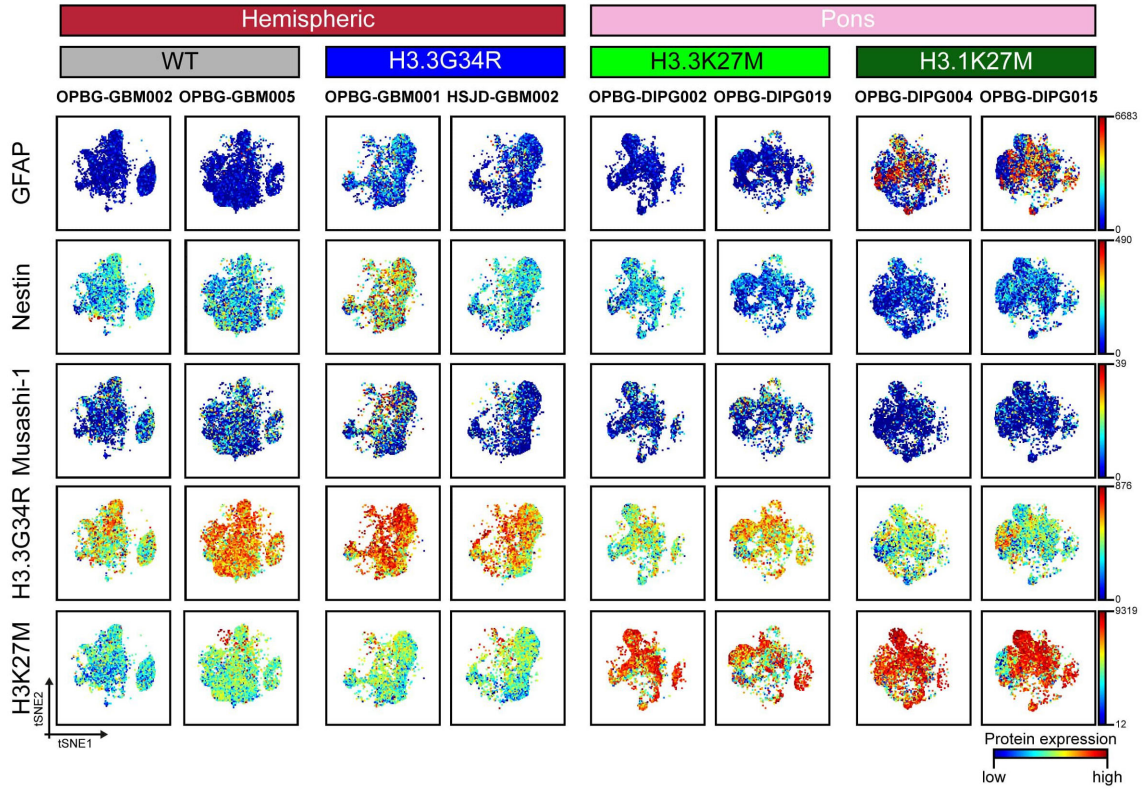

FIGURE 3
CyTOF single-cell analysis of intracellular antigen in PDHGG patient-derived cell lines. t-SNE maps showing the expression of 5 intracellular markers (GFAP, Nestin, Musashi-1, H3.3G34R and H3K27M) in each of the eight different PDHGG patient-derived cell lines analyzed through the mass cytometry technique. The color gradient refers to the intensity of the expression of the considered marker, in a blue to red scale indicating low and high intensity respectively.

heterogeneity was more evident for some markers such as the cell adhesion molecule CD34 and the α3 and β1 integrins (CD49c and CD61 respectively) which were expressed only by highly restricted subpopulations of some PDHGG patient-derived cell lines. For example, CD49c resulted to be specifically expressed by a group of cells whose presence was particularly highlighted in histone wild-type patient-derived cell lines (Figure 2). This result confirms the notion of the existence of intra-tumor heterogeneity for surface marker expression within PDHGG patient-derived cell lines.

We then looked at the expression of specific markers which are predicted to be differentially expressed by our model and tumor subtypes and that includes the two histone variants (H3K27M and H3.3G34R) as well as some stem and differentiation markers (Figure 3). Musashi-1 was hardly detected (maximum detection at 39) across all the tested cell lines while Nestin was diffusely expressed in the hemispheric patient-derived cell lines and, in particular, in the H3.3G34R cell line, OPBG-GBM001 (Figure 3). A noteworthy observation was highlighted for the glial differentiation marker GFAP, which resulted to be highly caught (maximum detection at 6683) in our conditions but exclusively in the two H3.1K27M patient-derived cell lines. Our analysis of intracellular antigens was enriched with specific custom conjugated antibodies targeting the histone mutants H3K27M and H3.3G34R which are useful to identify the specific patient-derived cell lines affected by these mutations, and to confirm, at the protein level, the histone molecular status also defined by Sanger sequencing analysis (Figure S1). However, while H3K27M antibody was highly specific, only targeting H3.3 and H3.1 histone mutant cells, H3.3G34R antibody was not so exclusive when used for CyTOF analysis, binding all the patient-derived cell lines regardless of the molecular subgroup to which they belong (Figure S2). This was particularly observed in the case of wild-type cell lines, for which a non-specific expression of the H3.3G34R antigen was observed.

As anticipated above, by looking at the overall expression of the surface and intracellular antigens targeted by our antibody panel, we could observe that some of the PDHGG patient-derived cell lines were particularly enriched in the expression of specific antigens in comparison to other cell lines included in the analysis. In order to investigate the level of inter-tumor heterogeneity by single-cell mass cytometry, we considered

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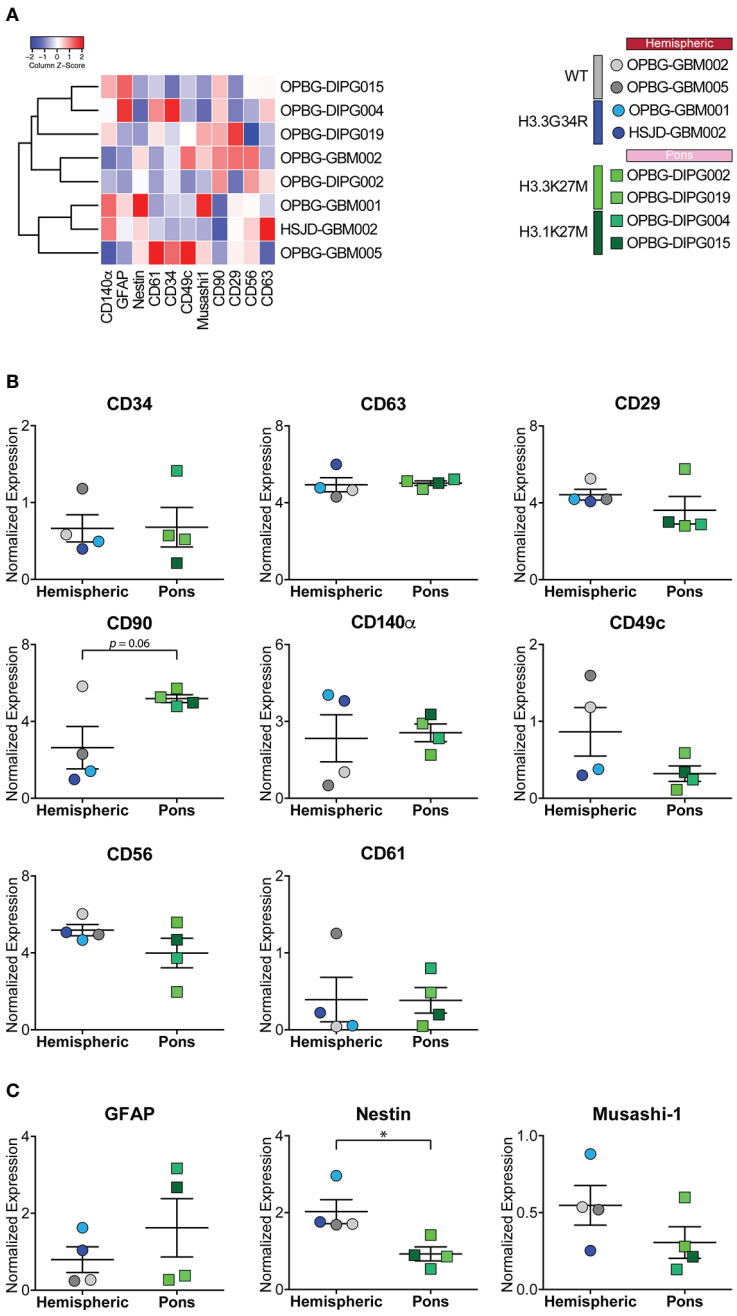

FIGURE 4
Marker expression analyzed through CyTOF technique in PDHGG patient-derived cell lines. **(A)** Heatmap summarizing the expression of the analyzed cell markers in the eight PDHGG cell lines. **(B)** Scatter dot plots showing the normalized expression of the indicated surface **(B)** and intracellular **(C)** markers in hemispheric and pontine PDHGG patient-derived cell lines. Each shape of the scatter dot plot indicates a different tumor location (round for hemispheric, square for pontine) while the color coding refers to the cell line mutational subgroups (see the key legend). *$p < 0.05$.

different subgroups of PDHGG patient-derived cell lines on the basis of their tumor location (hemispheric *versus* pontine) and their histone status (WT, H3.3G34R, H3.3K37M and H3.1K27M) and measured the expression of the surface and intracellular markers (Figure 4). By doing so, we were not able to define a specific pattern of expression for some markers, such as CD63, CD61 and CD34 whose expression tended to be similar in the defined subgroups. However, some relevant differences emerged between the subgroups. We highlighted that the neural and glioma cancer stem cell marker CD56 (36, 47, 48)

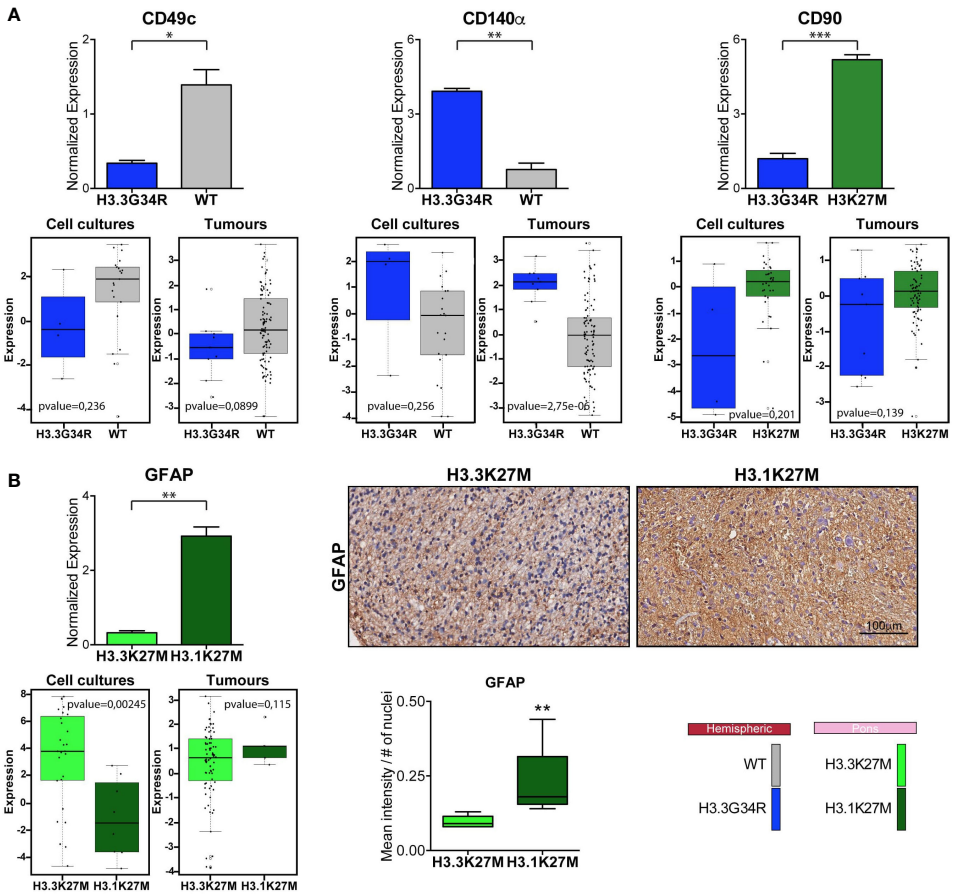

FIGURE 5
Comparison of marker expression in different molecular PDHGG patient-derived cell lines. **(A)** Bar plots showing the comparison between the H3WT *vs* H3.3G34R and H3K27M *vs* H3.3G34R PDHGG molecular subgroups relative to the expression of the indicated marker. The data on the top refer to the marker normalized expression obtained from mass cytometry data analysis while the plots on the bottom were obtained from RNA seq analysis on both patient-derived cell cultures (n=68) and tumor tissues (n=133). Data are represented as mean ± SEM. **(B)** Bar plots relative to the expression of GFAP marker in the H3.3K27M *vs* H3.1K27M PDHGG molecular subgroups obtained from CyTOF and RNA seq analysis. Representative images of GFAP immunohistochemistry on H3.3K27M and H3.1K27M PDHGG FFPE tissue slides together with the relative quantification of GFAP signal intensity normalized on the number of nuclei (n=5 for H3.1K27M; n=6 for H3.3 K27M). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

and the integrin β1, CD29 (40–42) were more uniformly expressed within the hemispheric subgroup, regardless of their histone alterations (Figures 4A, B). On the contrary, two of the analyzed markers resulted to be associated with a specific histone alteration occurring in the hemispheric subgroup. For one of these, the integrin-α3, CD49c (40), we noticed a higher expression in the two histone wild-type cell lines compared to the other molecular/locational subgroups (Figures 4A, B). For the CD140α marker, also known as PDGFRα, which is widely expressed in the brain but often amplified and/or overexpressed in brain cancer cell lines (36, 45, 46), our analysis showed that it was specifically associated with the H3.3G34R histone alteration of the hemispheric subgroup (Figures 4A, B). Moreover, we observed that the mesenchymal marker CD90 (43, 44) was homogenously expressed by the pontine PDHGG patient-derived cell lines subgroup and its expression resulted to be higher especially when compared to the hemispheric H3.3G34R patient-derived cell lines. In addition, the expression level of the astroglial differentiation marker GFAP was clearly higher in the H3.1K27M histone mutant PDHGG patient-derived cell lines.

Next, we focused on the markers for which a clear pattern of expression was observed between specific locational/molecular subgroups and looked at the gene expression level for these makers on a wider panel of patient-derived cell cultures (n=68) and tumor tissues (n=133) profiled by bulk sequencing (Figure 5). CD49c, which, on single-cell mass cytometry data was more highly expressed in hemispheric H3WT patient-derived cell lines, and CD140α which on the contrary was specifically associated with H3.3G34R, appear to have the same trend also on RNA expression level for both primary-

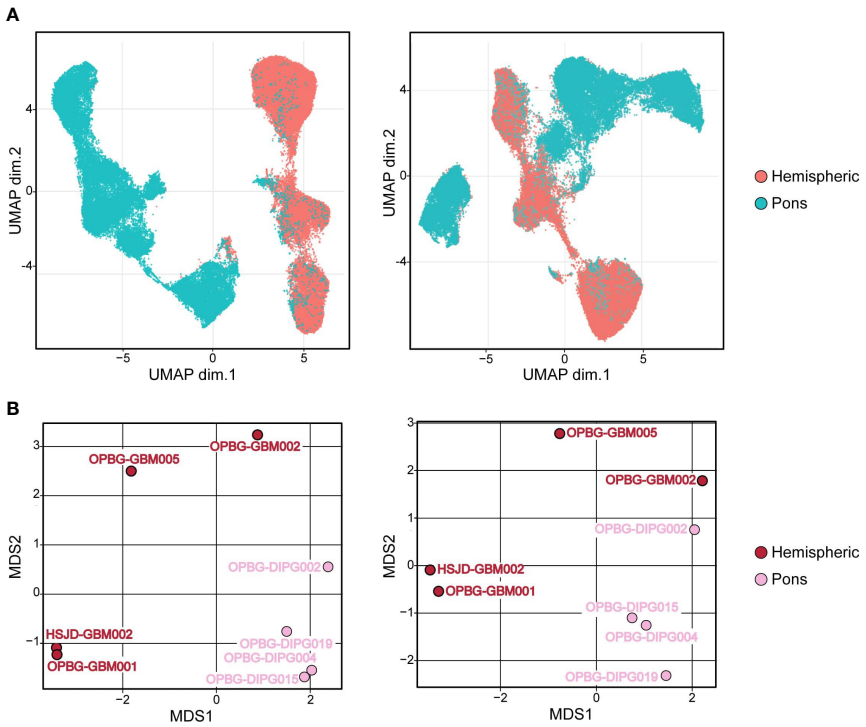

FIGURE 6
Hemispheric and pons patient-derived cell line separation. **(A)** UMAP projections and **(B)** Multidimensional Scaling plot (MDS) of PDHGG patient-derived cell lines obtained by including (left) or not (right) H3.3G34R and H3K27M histone variants in the relative analysis performed on single-cell mass cytometry data. The color refers to the locational subgroup to which the PDHGG patient-derived cell lines belong (hemispheric or pons, see the relative key legend).

derived cell cultures and tumor tissue samples (Figure 5A). CD90, based on the CyTOF data is more highly expressed in H3K27-altered patient-derived cell lines compared to the H3.3G34R mutant cell lines (Figure 5A), appear to have a similar trend at the RNA expression level. While GFAP was more strongly expressed in H3.1K27M compared to H3.3K27M mutant cell lines by CyTOF analysis, the same association was not confirmed at the RNA level (Figure 5B). However, the IHC staining performed on FFPE patient tissue sections, validated the mass cytometry data, showing a higher expression of GFAP at protein level in H3.1K27M tumors compared to H3.3K27M.

3.4 UMAP analysis on PDHGG patient-derived cell lines

Next, in order to define specific cell clusters and address the cell similarity in PDHGG patient-derived cell lines, we created a two-dimensional graph using the dimensionality reduction algorithm uniform manifold approximation and projection (UMAP). To compute UMAP, we specified all the markers listed in Table 1, to be considered for the algorithm, including the expression of the H3.3G34R and H3K27M mutant histones. The resulting UMAP projections showed that the cells belonging to the same locational subgroup, hemispheric and pons, clustered closer although a minimal degree of overlap between hemispheric and pons patient-derived cell lines was observed (Figure 6A, right panel). When including H3.3G34R and H3K27M histone alterations in the UMAP algorithm settings, the separation between hemispheric and pons patient-derived cell lines was even more clear (Figure 6A, left panel). These results suggest that the histone mutational status may have an impact on the determination of the cell antigenic profile but it also suggests that it is not the unique discriminating factor. In fact, by performing a multidimensional scaling (MDS) analysis we show that cells belonging to the same locational and molecular subgroup cluster quite closer already when the mutational alterations H3.3G34R and H3K27M were not taken into account for the analysis (Figure 6B, right panel).

In order to avoid any misinterpretation deriving from a low specificity of the H3G34R antibody, we next focused on the UMAP results that were obtained without including H3G34R and H3K27M histone variants in the analysis. The existence of 10 different clusters was described in the analyzed PDHGG cell lines and, as anticipated, they showed a minimal overlap between hemispheric and pontine subgroups as displayed on

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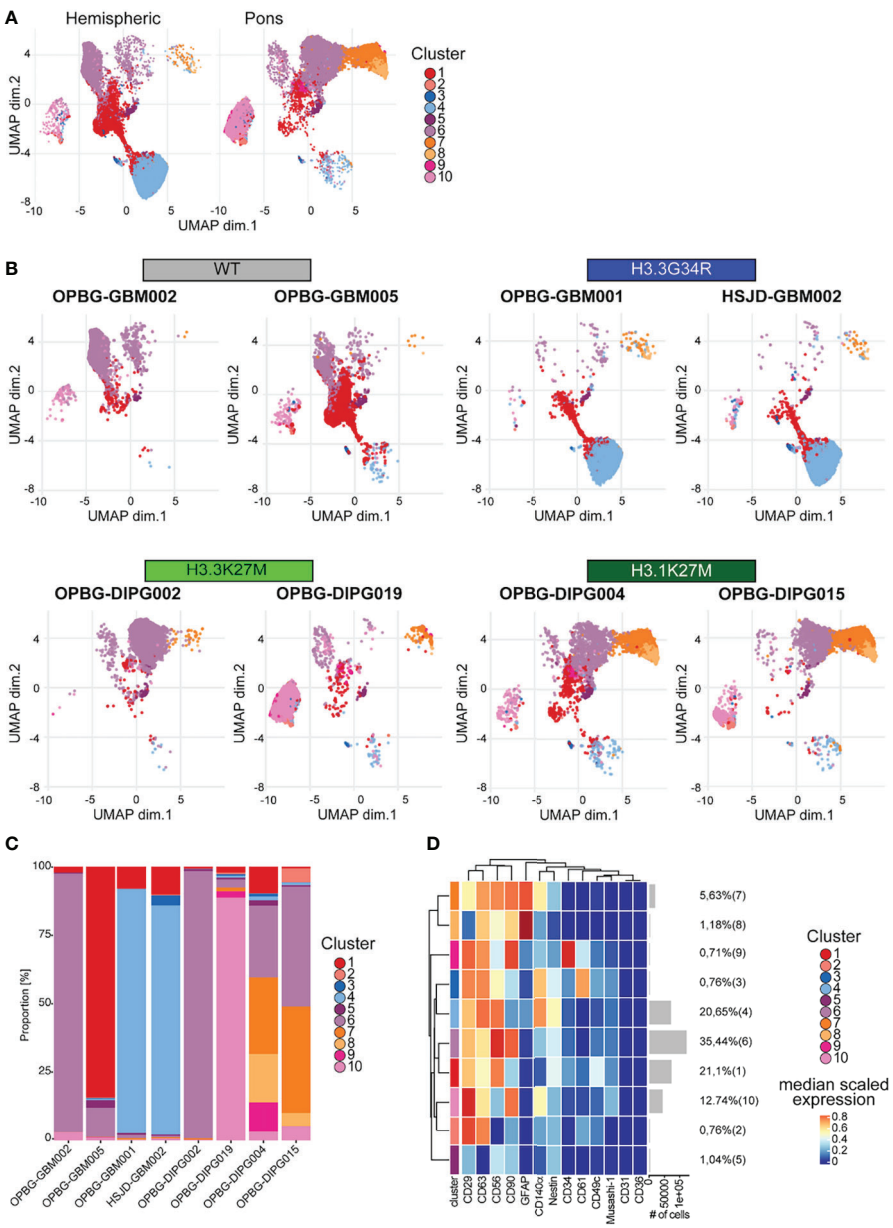

FIGURE 7
UMAP analysis. **(A)** UMAP plots of the hemispheric and pons cell lines colored according to the identified clusters. **(B)** UMAP plots for each of the analyzed cell lines, colored according to the identified cluster. **(C)** Bar plots representing the abundance of each of the clusters identified in each cell lines. **(D)** Heatmap summarizing the antigenic profile of each of the identified cluster.

the UMAP (Figure 7A). Moreover, patient-derived cell lines belonging to the same mutational subgroup show a high degree of overlap, even if the expression of the mutant histones was not taken into consideration for the analysis (Figure 7B). Focusing on the analysis of UMAP cluster composition, the two H3.3G34R mutant cell lines were highly uniform and largely characterized by cluster 4, which was mainly distinguished by the co-expression of CD29, CD63, CD56 and CD140α (Figures 7B–D and Supplementary Figure S3). The subcomposition of the cell lines belonging to the H3.1K27M molecular subgroup was also comparable, with cluster 6 and cluster 7 being the predominant clusters for both OPBG-DIPG004 and OPBG-DIPG015 cell lines. Cluster 7 was characterized by the co-expression of GFAP at higher lever, CD90, CD63 and CD56, while cluster 6 was identified by a higher expression level of CD56 and CD90 and, at a lower level,

TABLE 2 List of the cell antigens used together with the molecular function and reported expression.

Antigen	Other Name	Function	Expression	Reference
CD31	PECAM-1	Cell Adhesion, Angiogenesis	Endothelial Cells Glioma cancer stem cells	(35)
CD34	Hematopoietic Progenitor Antigen	Cell Adhesion	Progenitor Cells Cancer Stem Cells Mesenchymal-like Cells	(36)
CD63	Tetraspanin-30	Cell Receptor; IntegrinActivation and Signaling	Cancer Exosomes	(37)
CD36	Coll-1 Receptor	Angiogenesis, Cell Receptor	Endothelial Cells Cancer stem cells	(38, 39)
CD29	Integrin β-1	Cell Receptor	Cancer Cells	(40–42)
CD90	Thy-1	Cell-Cell/Cell-Ligand Interaction	Mature Neurons Mesenchymal Cancer Stem Cells	(43, 44)
CD140α	Platelet-Derived Growth Factor; PDGFRα	RTK involved in Proliferation, Survival and Migration	Widely Expressed in Brain Cancer Cells	(36, 45, 46)
CD49c	Integrin α-3	Cell Receptor	Cancer Cells	(40)
CD56	N-CAM1	Cell Adhesion	Neural Lineage Glioma Cancer Stem Cells	(36, 47, 48)
CD61	Integrin β-3	Cell Receptor	Cancer Cells	(46, 49)
GFAP	Glial Fibrillary Acid Protein	Mechanical and Cell Strength	Astrocytes Glioma Cells	(46)
Nestin	–	Survival and Proliferation	CNS Stem Cells	(41, 46, 50, 51)
Musashi-1	RNA-binding protein	mRNA Expression Regulation	CNS Stem Cells	(51)
H3.3G34R	–	Histone Mutation	DHG-G34	(52)
H3K27M	–	Histone Mutation	DMG-K27, pilocytic astrocytoma, and glioneuronal tumors	(53)

of CD29. On the contrary, the subcomposition of the histone wild type and H3.3K27M cell lines differed between the two cell lines within each group. In fact, the OPBG-GBM002 Histone WT cell line was dominated by cluster 6, whereas OPBG-GBM005 WT cell line was enriched in cluster 1, identified by the expression of CD56 and CD29, although cluster 6 was also present. For the DMG-K27 subgroup, OPBG-DIPG002 was distinguished by cluster 6 too, while OPBG-DIPG019 was mainly dominated by cluster 10, identified by the expression of CD29, at high level, CD90 and, at a lower level, CD63 and CD140α (Figures 7B–D and Supplementary Figure S3).

4 Discussion

The intra and inter-tumor heterogeneity is a hallmark feature of PDHGG contributing to major failure for treatment options and resistance to therapies attempted so far (55–58). Moreover,

TABLE 3 Summary of the clinico-pathological data for the eight PDHGG primary patient-derived cell lines used for the study.

Patient cell line	Gender	Age (years)	Procedure	Diagnosis	Location	Mutation
OPBG-GBM002	M	11	Resection	PDHGG-WT	Hemispheric	WT
OPBG-GBM005	M	9	Resection	PDHGG-WT	Hemispheric	WT
OPBG-GBM001	M	12	Resection	DHG-H3G34	Hemispheric	H3.3G34R
HSJD-GBM002	M	14	Biopsy	DHG-H3G34	Hemispheric	H3.3G34R
OPBG-DIPG002	F	6	Biopsy	DMG-H3K27	Pons	H3.3K27M
OPBG-DIPG019	M	8	Biopsy	DMG-H3K27	Pons	H3.3K27M
OPBG-DIPG004	M	6	Biopsy	DMG-H3K27	Pons	H3.1K27M
OPBG-DIPG015	F	4	Biopsy	DMG-H3K27	Pons	H3.1K27M

The panel shows the information regarding the patients' gender and age, as well as the type of procedure, diagnosis, tumor location and mutation status.

heterogeneity has also implication on the identification of reliable biomarkers useful for the diagnostic and prognostic stratification of the patients. In fact, molecular profiling studies highlighted that PDHGG tumors could be stratified into different subgroups according to their genetic signature, driving the clinico-pathological features of these malignancies (6, 13, 14). The inter-tumor heterogeneity which distinguishes PDHGG, contributes to identify subgroups different from one to the other, explaining the failure to find a unique treatment for all. Moreover, each individual tumor mass is characterized by different cell types that are organized in a well-defined functional network, in which normal brain cell compartments are also recruited, and that, by contributing to the aggressive phenotype of PDHGG malignancies and to their resistance to therapies, undermine even further the possibility to find a more effective therapeutic strategy for these deadly diseases. To date, most studies have focused their attention on genetic heterogeneity and just a few of them have analyzed phenotypic diversity. In this study, in order to enhance our comprehension on the inter- and intra-tumor heterogeneity underlying PDHGG tumors, we take advantage of the CyTOF technology to investigate the expression of multiple extracellular and intracellular markers at single-cell resolution. To this end, we adopted a panel of 15 antibodies, designed to capture the phenotypic plasticity of PDHGG cells by targeting antigens expressed by tumor and stem-like cells as well as by normal brain microenvironment components. In particular, we characterized the single-cell phenotypes of eight patient-derived tumor cell lines, carrying different genetic alterations and arising from two distinct locational compartments of the brain, the hemispheres and the pons. By applying this approach, we obtained clear evidence that PDHGG patient-derived cell lines consisted of heterogeneous cells exhibiting dissimilar antigenic profiles, with cells expressing markers at a high level and cells completely negative for the same antigens within the same patient-derived cell line. This intra-tumor heterogeneity was evident especially when looking at the expression of some markers such as CD49c (integrin α3), CD61 (integrin β3), and CD34, which were restricted to distinct subclones. CD49c, CD61 and CD34, are all hallmarks of tumor aggressiveness: CD49c, by cooperating with EGFR, has been shown to contribute in driving tumor cell motility and invasion especially in histone WT patient-derived cell lines (59); CD61 is one of the most widely studied members of the integrin family, involved in tumor progression (60, 61) while CD34 overexpression in glioma tissues was closely associated with higher WHO grade (III+IV) (62). These observations suggest the possibility that our analysis enables the identification of more rare subclones, potentially with a more aggressive phenotype, which is also in line with what we have previously shown (12). At the inter-tumor level, according to previous observations on glioblastoma (42, 61, 63), our analysis shows that all patient-derived cell lines analyzed express the neural cell adhesion molecule CD56, the integrin β1 CD29 and its activator CD63, even if a great variability between each patient-derived cell line was observed. The mesenchymal marker CD90, a glycoprotein known to be expressed in glioblastoma and associated with an adhesion/migration gene signature and invasive tumor features (43, 44, 64), was upregulated in the pontine tumor, regardless the molecular alterations. This association was significantly observed at protein level. Interestingly, an inter-tumor heterogeneity between cell lines belonging to the same locational subgroup also arises from our study. In this regard, great differences were observed in the expression of PDGFRα (CD140α) marker which is frequently mutated/amplified in PDHGG tumors (14, 65, 66). Although there is a heterogeneity that emerges from the single-cell analysis within each cell line, our data showed a predilection of CD140α for the DHG-H3G34 subgroup, particularly when compared to the histone WT hemispheric counterpart, both at protein and RNA level. Our findings are in line with recent data pointing to the implication of PDGFRα alteration in DHG-H3G34 (67). The critical role of this marker has been demonstrated in the co-option with G34R/V, promoting malignant gliogenesis in these tumors (25). Interestingly, both of our DHG-H3G34 mutant cell lines also carry a mutation in PDGFRα. Within the DMG-K27 subgroup, the differentiation marker GFAP was more highly expressed, only at protein level, in H3.1K27M subgroup, suggesting a more pronounced astroglial differentiated phenotype for this tumor subgroup, in line with what reported also in Castel et al., 2015 (29, 53). Most of the antibodies included in the panel were already conjugated with metal. However, in order to refine our analysis by unequivocally identifying tumor cells, we specifically customized two histone variant antibodies, anti-H3.3G34R and anti-H3K27M. The antibody we have used in our study for the H3K27M mutation is commonly used in a reliable manner in routine diagnostic setting (4). Moreover, the same antibody, custom-conjugated has been used in a recently published work that, by employing the CyTOF technology, has investigated the epigenetic rearrangements due to H3K27M alteration in a panel of DMG-H3K27M mutant cell lines (68). Interestingly, Harpaz et al., have demonstrated the existence of two epigenetically distinct subpopulations in DMG-H3K27M mutant cell lines and suggest that these differences mirror the heterogeneous expression of the H3K27M oncohistone (68). While we confirmed that the antibody anti-H3K27M can be used in a robust manner, unfortunately, the H3.3G34R antibody, which was custom-conjugated and used for CyTOF analysis for the first time in this study, did not prove accurate, due to its poor specificity. In our study, this antibody lacked specificity. In fact, besides recognizing the H3.3G34R mutant cells, it also binds H3WT and, to a minor extent, H3K27M mutant cells. However, being adopted for the first time in such a study, even if its functionality in mass cytometry analysis is not optimal and would require improvements, its specificity in IHC testing has been already questioned by others who concluded that the H3.3G34R antibody is not highly predictive for the presence of G34R/V mutation and that confirmation by sequencing is mandatory (52, 69). Although the anti-H3.3G34R antibody functionality was suboptimal, when we performed the UMAP and MDS analysis, the eight patient-derived cell lines that were tested in our mass

cytometry experiments clearly separated in two subgroups when histone variants antibodies were not included in the analysis and, to a great extent, also when they were included. This observation suggests the hypothesis that patient-derived cell line antigenic profiles may be largely imprinted by their molecular alterations. In order to circumvent any alteration that could affect cell clustering due to the non-specific binding of the H3.3G34R antibody to cells, we decided to remove both histone variant antibodies from the downstream analysis. Interestingly, UMAP analysis shows that the hemispheric H3G34R and the pontine H3.1 patient-derived cell lines were more homogenous than the hemispheric WT and pontine H3.3 lines in terms of cell cluster subcomposition. The hemispheric H3G34R were mainly populated by cluster 4 ($CD56^+$, $CD63^+$, $CD140\alpha^+$, $CD29^+$, $Nestin^{int}$) while the pontine H3.1 were mainly distinguished by cluster 6 ($CD56^+$, $CD90^+$, $CD29^+$, $CD63^{int}$) and 7 ($GFAP^+$, $CD90^+$, $CD56^+$, $CD63^+$, $CD29^{int}$, $CD140\alpha^{int}$).

Our mass cytometry analysis on PDHGG primary patient-derived cell lines has pointed out toward potential biomarkers given by the association of specific antigens to distinct tumor subgroups. Although cell cultures are only partially recapitulating the complexity and the heterogeneity of PDHGG patient tumors, we have shown that our mass cytometry data can be validated by IHC analysis on patient tissue sample as exemplified for GFAP staining. Additional work on further validation on patient tissue sample may demonstrate the utility of the antigenic profiles we have identified on the primary cell lines by mass cytometry analysis.

Although our study is limited by a relatively small number of antibodies and also a small number of primary patient-derived cell lines, it is the first CyTOF study of this kind across the heterogeneous repertoire of the diffuse pediatric-type high-grade glioma family, and highlighted the opportunity to apply the mass cytometry technology to this complex biological context with its relevant potential and limitations.

In the future, the use of a larger panel of metal-tagged antibodies will further highlight the multidimensional potential of mass cytometry for PDHGG. This will require though a more extensive work for the *ad-hoc* customization of specific antigens of interest, for which there still is a lack of commercially available metal-conjugated antibodies, especially for brain and brain tumors. In particular, for PDHGG it will be useful to generate focused antibody panels for pathology driven biomarkers or to study specific cellular processes such as invasion/migration, and/or to focus on specific pathways in relation to potential therapeutic treatments.

We believe that the mass cytometry technology and its multidimensional analysis capability may contribute to further advance the field of PDHGG. It can be used to comprehensively characterize patient-derived models to determine how certain antigenic profiles are retained in different culture conditions (2D *vs* 3D and organoid cultures). Moreover, the use of focused metal-tagged antibody panels may be employed to study, at single-cell level, how primary patient-derived cells respond to therapeutic approaches of interest, highlighting the identification of biomarkers, allowing to follow the dynamic modulation of multiple markers and their functional states, comparing different conditions (*e.g.* pre and posttreatment) and identifying unique cell populations responsive and/or resistant to treatment. Finally, the effort to generate a custom-conjugated antibody panel for the PDHGG and the brain tumor-immune microenvironment will offer a more expanded vision on the complexity of these tumors with more advanced CyTOF based imaging mass cytometry technology for studying patient tissue samples *in situ* at single-cell level.

In conclusion, mass cytometry analysis has shown that PDHGG patient-derived cell lines are comprised by cells having different antigenic profile at both intra- and inter-tumor level. Our study opens to the possibility of employing tumor cell antigens, identified through mass cytometry analysis, as predictive biomarkers for molecular/locational PDHGG subgroup and for patient stratification.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Ethical Committee of the Bambino Gesù Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

Conceptualization, LLP, CF, and MV; methodology, LLP, CF, LP, AP, GP, ALM, YG, and SR; validation, LLP; formal analysis, LLP, AP, ALM, and YG; writing—original draft preparation, LLP; writing—review and editing, LLP, CF, AP, LP, GP, ALM, YG, AC, ANM, CJ, GC, FL, and MV; supervision, MV; reagents, cases, data and/or clinical annotation: CF, SR, AMC, AC, ANM, CJ, and GC; funding acquisition, FL and MV. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by Children with cancer UK Grant 16-234 (MV); DIPG Collaborative (MV); Italian Ministry of Health "Fondo 5x1000" (FL and MV). Lucia Lisa Petrilli was supported by Fondazione Umberto Veronesi (2022) and by AIRC fellowship for Italy (2020). We further acknowledge Fondazione Heal.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1016343/full#supplementary-material

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frontiers | Frontiers in Oncology

TYPE Brief Research Report
PUBLISHED 17 January 2023
DOI 10.3389/fonc.2022.1000215

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 21 July 2022
ACCEPTED 28 December 2022
PUBLISHED 17 January 2023

CITATION
Ariagno S, Ragoonanan D, Khazal S, Mahadeo KM, Cisneros GS, Zinter MS, Blacken RA, Mohan G, Lehmann LE, Ferdjallah A, Mara KC and Kohorst MA (2023) Prior COVID-19 infection may increase risk for developing endothelial dysfunction following hematopoietic cell transplantation.
Front. Oncol. 12:1000215.
doi: 10.3389/fonc.2022.1000215

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Prior COVID-19 infection may increase risk for developing endothelial dysfunction following hematopoietic cell transplantation

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Endothelial dysfunction underlies many of the major complications following hematopoietic cell transplantation (HCT), including transplant-associated thrombotic microangiopathy (TA-TMA), veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS), and engraftment syndrome (ES). Emerging evidence similarly implicates endothelitis and microangiopathy in severe COVID-19-related multi-system organ dysfunction. Given the overlap in these two illness states, we hypothesize that prior COVID-19 infection may increase risk for HCT-related endotheliopathies. This retrospective, multicenter study included patients aged 0-25 years who underwent autologous or allogeneic HCT for any indication between January 1, 2020 and September 21, 2021, with close attention to those infected with COVID-19 in either the six months prior to transplant or twelve months following transplant. Incidences of TA-TMA, VOD/SOS, and ES were compared among patients with COVID-19 infection pre-HCT and post-HCT, as well as with historical controls who were never infected with SARS-CoV-2. Those who underwent HCT following COVID-19 infection displayed significantly increased rates of TA-TMA compared to those who were never infected. Additionally, our data suggests a similar trend for increased VOD/SOS and ES rates, although this did not reach statistical significance. Therefore, a history of COVID-19 infection prior to undergoing HCT may be a nonmodifiable risk factor for endothelial-related complications following HCT. Further studies are warranted to better clarify this relationship among larger cohorts and in the era of the Omicron SARS-CoV-2 variants.

KEYWORDS

COVID-19, hematopoietic cell transplant, TA-TMA, VOD/SOS, engraftment syndrome, endothelial dysfunction

1 Introduction

While scientific knowledge regarding the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has expanded rapidly since the beginning of the coronavirus disease 2019 (COVID-19) pandemic, unanswered questions remain pertaining to the impact of this illness on medically unique populations. Pediatric patients undergoing hematopoietic cell transplantation (HCT) are a medically complex group in which the impact of SARS-CoV-2 infection is not well understood. While pediatric patients in general seem to be at lower risk for severe disease from COVID-19, adults undergoing HCT appear to be at increased risk for COVID-19-related severe disease and mortality (1–3).

Data characterizing the experience of this unique pediatric population is limited and predominately describes patients infected following HCT. Lucchini et al. recount the UK experience, in which the majority of their post-HCT pediatric patients who became infected with SARS-CoV-2 had mild illness, although one of the nine patients did develop severe COVID-19 infection with cytokine-release syndrome (4). A series of more severe cases was described by Barhoom et al., where they detailed four pediatric patients who contracted COVID-19 following HCT, three of whom required endotracheal intubation and ICU level care and two of whom died as a result of the infection (5). The largest study to date includes 62 children infected with SARS-CoV-2 following HCT, with 10% of those patients requiring ICU level care (6). COVID-19-specific mortality among this cohort was 5% (6). In total, most of the available literature highlights COVID-19 infection months following transplant, with little data available regarding patients who acquire SARS-CoV-2 prior to or immediately surrounding HCT. Additionally, no available literature describes the relationship between COVID-19 infection and HCT-specific complications or outcomes.

Mounting evidence suggests that severe disease from SARS-CoV-2 is largely mediated by systemic endothelial injury (7, 8). Analysis of biopsy samples from surviving and postmortem patients with severe COVID-19 infection reveals endothelial destruction in all major organ systems (9–11). Under normal conditions, endothelial cells regulate vascular tone, inflammatory cascades, vessel permeability, and the balance between prothrombic and antithrombic states. In the setting of SARS-CoV-2 infection, endothelial dysfunction likely arises from a combination of two factors – direct viral invasion of endothelial cells and cytokine hyperactivation initiated by pulmonary COVID-19 infection (10–12). Subsequently, diffuse vascular leakiness and interstitial edema, microvascular and macrovascular thromboembolic events, and overwhelming immune activation lead to multisystem organ dysfunction/failure (8, 10, 13).

The effect of COVID-19 infection on endothelial function may persist long after the acute infection has resolved. A study by Chioh et al. demonstrated that there were persistent cellular and biochemical markers of endothelial injury and cytokine hyperactivation among convalescing patients (14). Additionally, in prospective studies examining endothelial function as estimated by brachial artery flow-mediated dilation, multiple groups have demonstrated persistent endothelial dysfunction up to six months following COVID-19 infection (15, 16). These authors hypothesize that this continued endothelial dysfunction may predispose patients with a history of COVID-19 infection to thromboembolic events. Indeed, clinical data is accumulating that some adult patients may experience ongoing risk of vascular complications for weeks after acute SARS-CoV-2 infection (17, 18).

The systemic endothelial dysfunction seen in severe SARS-CoV-2 infection shares a similar pathophysiology with the endotheliopathies seen following HCT (19, 20). This collection of post-HCT complications include transplant associated thrombotic microangiopathy (TA-TMA), veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS), engraftment syndrome (ES), graft-versus-host disease (GvHD), idiopathic pneumonia syndrome/diffuse alveolar hemorrhage (IPS/DAH), and thrombosis/coagulopathy. Factors that contribute to endothelial injury in HCT settings include high-intensity conditioning chemotherapy, total body irradiation, certain underlying oncologic diagnoses, degree of alloreactivity, and GvHD prophylactic agents such as calcineurin inhibitors (21). In each of these complications, systemic or organ-specific endothelial damage occurs, leading to increased vascular permeability, decreased regulation of vascular tone, activation of coagulation factors, and excess inflammatory/cytokine signaling (21).

Given these shared mechanisms, as well as the lingering nature of the endothelial dysfunction following COVID-19 infection, we hypothesize that patients with SARS-CoV-2 infection in the time period immediately pre- or post- HCT may be at increased risk of developing HCT-related complications mediated by endothelial dysfunction. Therefore, in this study we assessed the incidence of transplant-related endotheliopathies in patients with COVID-19 infection pre-HCT and post-HCT compared to historical controls without documented SARS-CoV-2.

2 Methods

This multicenter study evaluated patients across four geographically diverse academic pediatric centers. All patients aged 0-25 years who underwent autologous or allogeneic HCT for any indication from January 1, 2020 to September 21, 2021 were included. The study group was comprised of all patients with a laboratory confirmed case of COVID-19 in either the six months prior to or twelve months following HCT. Clinical variables of interest were collected *via* retrospective review of the medical record and stored on a de-identified, secure database.

Data was summarized using frequencies and percentages for categorical data and medians with either ranges or interquartile ranges for continuous data. Data was compared between groups (COVID-19 pre-HCT vs COVID-19 post-HCT) using a Fisher's exact tests for categorical data, and a Wilcox rank sum test for continuous data. All tests were two-sided, and p-values ≤ 0.05 were considered statistically significant. All analyses were performed using R version 4.1.2 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria).

This study was approved by the institutional review boards at each of the participating centers. Patient consent was not required given the de-identified and retrospective nature of the investigation.

3 Results

A total of 374 pediatric and young adult patients underwent HCT for any indication during the specified date range across the four sites. Ten patients contracted COVID-19 in the six months prior to HCT, and 13 contracted COVID-19 in the twelve months following HCT. Demographic characteristics of the infected patients are in Table 1A. No significant differences in age, sex, race, pre-transplant performance score, or underlying pre-transplant diagnosis were found between the COVID-19 Pre-HCT group and COVID-19 Post-HCT group.

Clinical data regarding HCT variables were compiled for patients who contracted COVID-19 (Table 1B). Transplant type, number of lifetime transplants, stem cell source, intensity of conditioning regimen, GVHD prophylactic agents, and exposure to serotherapy were similar between the groups. Patients in the COVID-19 Pre-HCT group were significantly more likely to have undergone haploidentical or mismatched transplantation, whereas patients in the COVID-19 Post-HCT group were more likely to have undergone matched transplantation.

COVID-19 infection characteristics were compiled (Table 2). Median date of COVID-19 diagnosis for the Pre-HCT group was day -107.5 (range: -73 to -205); the Post-HCT median infection date was day +206 (range: +54 to +345). Symptoms of COVID-19 infection were largely similar between groups, except for gastrointestinal symptoms, which were more likely to be reported in the Pre-HCT group. Rates of asymptomatic infection were similar between groups (20% vs. 31%, $p = 0.66$), as was need for intensive care (10% vs. 15%, $p = 0.99$).

Incidence of TA-TMA, VOD/SOS, and ES were compared between patients infected with COVID-19 Pre- and Post-HCT, as well as to the larger group of historical controls who were never infected with COVID-19 (Table 3). Patients in the Pre-HCT group were significantly more likely to experience TA-TMA as compared with patients who were never infected with COVID-19 (40% vs. 4.6%, $p = 0.003$). Several other non-statistically significant trends were noted as well. Incidence of TA-TMA may be higher among the Pre-HCT patients as compared to Post-HCT patients (40% vs. 8%, $p = 0.20$). VOD/SOS may occur more frequently among the Pre-HCT patients, as compared to the Post-HCT patients (30% vs. 8%, $p = 0.42$) and the non-COVID patients (30% vs. 14.8%, $p = 0.42$). ES displayed similar non-statistically significant trends. Incidence among the Pre-HCT patients may be increased as compared to the Post-HCT patients (20% vs. 0%, $p = 0.35$) and the non-COVID patients (20% vs. 8.8%, $p = 0.35$). Rates of VOD/SOS and ES were lowest in the Post-HCT patients (8%, 0%), which may be a reflection of small sample size.

Individual case details for the 10 patients in the COVID-19 Pre-HCT group are outlined in Table 4. Two patients died (20%); one death was attributed to multi-system organ failure in the setting of multiple transplant-related endotheliopathies. The other death was due to multi-system organ failure from both COVID-19 infection and multiple transplant-related endotheliopathies. Among the patients who were in the COVID-19 Post-HCT group, two patients died (15.4%). Neither death was attributed to COVID-19 infection or transplant-related endotheliopathy.

4 Discussion

In this study, we sought to understand the impact of COVID-19 infection on pediatric HCT outcomes, particularly transplant-associated endotheliopathies. Among this small group of pediatric and young adult patients, those who underwent HCT following COVID-19 infection displayed significantly increased rates of TA-TMA compared to those who were infected after transplant or historical controls who were never infected. Additionally, our data suggests a similar trend for increased VOD/SOS and ES rates, although this did not reach statistical significance. We do acknowledge that rates of VOD/SOS and ES were lowest in our cohort of patients who contracted COVID-19 following HCT, however we suspect this is due to small sample size and low statistical power rather than a true protective effect. Finally, for both patients from the COVID-19 Pre-HCT cohort who died, multiple identified endotheliopathies contributed to mortality.

To our knowledge, no other studies have reported on HCT-associated outcomes in pediatric patients with a history of prior COVID-19 infection. However, there are a few cases in the literature that support the pathophysiologic link between transplant endothelitis and COVID-19 endothelial dysfunction. Among the patients described by Lucchini et al., one patient who had mild illness from COVID-19 infection on day +138 went on to develop TA-TMA, in the absence of other commonly accepted TA-TMA risk factors (4). The authors suggest that COVID-19 infection was the suspected trigger for endothelial derangement in this patient. In the same cohort, the only patient who experienced severe illness and cytokine release syndrome from COVID-19 infection had undergone HCT for sickle cell anemia, specifically due to cerebrovascular disease which may suggest pre-existing endothelial dysfunction (4). Additionally, one patient reported by Barhoom et al. who developed COVID-19 infection on day +27 of transplant course developed TA-TMA within days of the infection and ultimately died as a result of this complication (5).

Evidence to support the increased risk of TA-TMA among transplant recipients who experience COVID-19 infection reaches beyond the realm of HCT. Two cases of TMA concurrent with COVID-19 infection in renal transplant recipients have been reported. The first describes an adult patient who was nine years post-allograft receipt and developed TMA 11 days following COVID-19 diagnosis; other known causes of TMA were ruled out. His course was also characterized by multisystem organ dysfunction and myocardial infarction, suggestive of global endothelial dysfunction (22). The second patient contracted COVID-19 three months following renal transplantation; acute kidney injury, hemolytic anemia, and coagulopathy were the presenting features of her COVID-19 diagnosis. Renal biopsy confirmed the diagnosis of TMA, with SARS-CoV-2 infection named as the suspected cause (23).

The shared pathogenic mechanism between severe COVID-19 infection and post-HCT endotheliopathies may be rooted in excess complement activation. SARS-CoV-2 infections have been shown to cause aberrant complement activation *via* the classical, lectin, and alternative complement pathways (24, 25). Genetic differences in the individual components of the complement system have been proposed as a contributing factor to varying illness severity

TABLE 1A Baseline demographic characteristic of HCT patients who contracted COVID-19 pre- and post-transplant.

	All COVID-19 Patients (N=23)	COVID-19 Pre-HCT (N=10)	COVID-19 Post-HCT (N=13)	p-value
Age, years , Median (IQR)	15.0 (5.3, 17.0)	14.6 (5.7, 16.0)	16.0 (5.0, 20.0)	0.71
Sex (% male)	61%	70%	54%	0.67
Race				0.2
White	48%	60%	38%	
Black or African American	4%	0%	8%	
More than one race	30%	40%	23%	
Unknown	17%	0%	31%	
Pre-Transplant Performance Score, Median (range)	90 (50-100)	90 (70-100)	90 (50-100)	0.63
Underlying Diagnosis				0.78
AML	30%	30%	31%	
ALL	17%	20%	15%	
MDS/MPN	9%	10%	8%	
Other acute leukemia	4%	0%	8%	
Hodgkin lymphoma	4%	0%	8%	
Solid tumor	9%	20%	0%	
SAA	4%	10%	0%	
Immune disorder	17%	10%	23%	
Other	4%	0%	8%	

TABLE 1B HCT variables of patients who contracted COVID-19 pre- and post-transplant.

	All COVID-19 Patients (N=23)	COVID-19 Pre-HCT (N=10)	COVID-19 Post-HCT (N=13)	p-value
Transplant Type				0.99
Allogeneic	83%	80%	85%	
Autologous	17%	20%	15%	
Transplant Number				0.99
First	78%	80%	77%	
Second	13%	10%	15%	
Third	9%	10%	8%	
Donor Type				0.02
MRD	17%	0%	31%	
MUD	17%	0%	31%	
Haploidentical	26%	50%	8%	
MMUD	17%	30%	8%	
UCB	4%	0%	8%	
Autologous	17%	20%	15%	
Stem Cell Source				0.81
Bone marrow	48%	40%	54%	

(Continued)

TABLE 1B Continued

	All COVID-19 Patients (N=23)	COVID-19 Pre-HCT (N=10)	COVID-19 Post-HCT (N=13)	p-value
PBSC	48%	60%	38%	
UCB	4%	0%	8%	
Conditioning Regimen				0.8
Myeloablative	70%	80%	62%	
RIC	26%	20%	31%	
Non-Myeloablative	4%	0%	8%	
GVHD Prophylaxis (may have received more than one)				
Ex-vivo T cell depletion	13%	20%	8%	0.56
CD34 selection	0%	0%	0%	–
PTCY + other	26%	30%	23%	0.99
PTCY alone	0%	0%	0%	–
TAC/CSA + MTX +/- other	26%	30%	23%	0.99
TAC/CSA + MMF +/- other	35%	60%	31%	0.22
TAC/CSA + other	9%	0%	15%	0.49
TAC/CSA alone	0%	0%	0%	–
Other	4%	0%	8%	0.99
None	13%	20%	8%	0.56
Serotherapy				0.57
ATG	30%	30%	31%	
Alemtuzumab	9%	0%	15%	
None	61%	70%	54%	

amongst patients with COVID-19. In particular, several polymorphisms of the MBL allele, which codes for the MBL protein of the lectin pathway, are known to cause excess complement activation and are associated with poor clinical outcomes in setting of severe infections (25). Similarly, excess complement activation is a contributing cause to post-HCT endothelial dysfunction, particularly TA-TMA. A number of pathogenic variants in a variety of complement factors have been identified in association with increased risk for TA-TMA (25–27). Further investigation is recommended to evaluate for unifying genetic predisposition for both conditions. Greater understanding of the genetic underpinnings for excess complement activation may eventually impact clinical guidance for increased screening, personalized transplant protocols, and therapeutic strategies for affected individuals.

Additionally, excess and prolonged cytokine release is likely a shared pathophysiologic mechanism that unifies severe COVID-19 illness and HCT-associated endotheliopathies. IL-6, IL-1β, and TNF-α have been named as key cytokines that become overexpressed in the setting of severe COVID-19 infection (28). Particularly for the pediatric population, these are key cytokines involved in the development of the multisystem inflammatory syndrome in children (MIS-C) that arises after COVID-19 infection. Specific therapeutic targets such as tocilizumab and anakinra are being investigated for their use in severe COVID-19 illness, due to their effects of modulating excess cytokine signaling. Likewise, IL-6 and TNF-α, in addition to a number of other cytokines, are excessively active in patients who have developed HCT-associated endothelial complications (21, 29). The use of targeted cytokine antagonists in preventing or treating HCT-associated endotheliopathies has been suggested in the literature, however to our knowledge has not been explored in a large-scale trial. Further investigation into cytokine signaling among patients undergoing HCT in close temporal relation to SARS-CoV-2 infection is warranted to better understand this relationship.

Two clinical applications of interest arise from these findings. First, our reported findings may suggest that prior COVID-19 infection could be a nonmodifiable risk factor for transplant-associated endotheliopathy. As such, institutions may consider implementing additional screening for affected patients throughout their transplant process. For example, we endorse the TA-TMA screening guidelines recommended by Dandoy et al., which includes twice weekly lactate dehydrogenase measurement and weekly urinalysis with urine protein creatinine ratio for the first 30

TABLE 2 SARS-Cov-2 infection details among HCT patients who contracted COVID-19 pre- and post-transplant.

	All COVID-19 Patients (N=23)	COVID-19 Pre-HCT (N=10)	COVID-19 Post-HCT (N=13)	p-value
Date of Diagnosis Relative to Day 0, Median (range)	120 (-205 to 345)	-107.5 (-73 to -205)	206 (54 to 345)	
COVID-19 Symptoms (may have had ≥ 1 symptom))				
Pyrexia	43%	60%	31%	0.22
Respiratory	65%	60%	69%	0.69
Cardiovascular	4%	0%	8%	0.99
Gastrointestinal	17%	40%	0%	0.024
Headaches	9%	20%	0%	0.18
Other neurological	9%	0%	15%	0.49
Coagulopathy	4%	10%	0%	0.43
Loss of taste and/or smell	0%	0%	0%	–
Asymptomatic	26%	20%	31%	0.66
Other	0%	0%	0%	–
ICU Level Care Required	13%	10%	15%	0.99
COVID-19 Directed Therapies (may have received more than one)				
Convalescent plasma	13%	30%	0%	0.068
Monoclonal antibody	9%	10%	8%	0.99
Remdesivir	30%	50%	15%	0.17
Steroid	26%	40%	15%	0.34
Tocilizumab	0%	0%	0%	–
Azithromycin	4%	10%	0%	0.43
Other antibiotic	17%	20%	15%	0.99
Other antiviral	0%	0%	0%	–
Other	13%	20%	8%	0.56
None	61%	50%	69%	0.42

days post-HCT. For patients undergoing HCT for non-malignant and non-urgent indications who contract COVID-19 prior to HCT, consideration may be given to delaying HCT. The appropriate interval for delaying HCT is not yet defined, and further investigation is required prior to formalizing this recommendation (30). Second, recognition of the overlapping risks between HCT-related endotheliopathies and COVID-19 endothelial dysfunction raises the question of shared therapeutics between the two diseases. Prior researchers have called for studies investigating the use of agents for VOD/SOS and TA-TMA for severe COVID-19. Both defibrotide

TABLE 3 Incidences of TA-TMA, VOD/SOS, and ES among HCT patients who contracted COVID-19 pre-transplant, post-transplant, or were never infected.

	COVID-19 Pre-HCT (n=10)	COVID-19 Post-HCT (n=13)	Non-COVID HCT Patients (n=351)	Pre vs Post p-value	Pre vs Non p-value	Post vs Non p-value
TA-TMA	40% (n=4)	8% (n=1)	4.56% (n=16)	0.2	0.003	0.47
VOD/SOS	30% (n=3)	8% (n=1)	14.81% (n=52)	0.42	0.42	0.7
ES	20% (n=2)	0% (n=0)	8.83% (n=31)	0.35	0.35	0.61

TABLE 4 Case details of all HCT patients who were infected with COVID-19 pre-transplant.

Patient #	Age (years)	M/F	Underlying Diagnosis	Transplant Type	Conditioning	GVHD Prophylaxis	COVID-19 Date of Diagnosis	COVID-19 Symptoms	COVID-19 Directed Therapies	Need for ICU level care for COVID-19	Coagulopathy	TA-TMA	VOD/SOS	Engraftment syndrome	Acute GVHD (Grade II-IV)	Chronic GVHD	Vital Status	Cause of death
1	5	M	Severe aplastic anemia	Allogeneic	RIC	T-cell depletion, MMF	-205	Pyrexia, GI, headaches	None	No	No	Yes	No	No	No	No	Alive	Not applicable
2	3	M	Neuroblastoma	Autologous	Myeloablative	None	-118	Asymptomatic	None	No	No	Yes	Yes	Yes	No	No	Deceased	Multi-system organ failure secondary to DAH, TA-TMA, VOD/SOS
3	14	M	Ewing sarcoma	Autologous	Myeloablative	None	-79	Asymptomatic	None	No	No	No	No	No	No	No	Alive	Not applicable
4	24	F	AML	Allogeneic	Myeloablative	T-cell depletion	-80	Respiratory	Remdesivir, steroids	No	No	No	No	No	No	No	Alive	Not applicable
5	16	M	ALL	Allogeneic	Myeloablative	PTCY, Tacrolimus, MMF	-134	Pyrexia, respiratory	Convalescent plasma, remdesivir	No	No	No	Yes	No	No	No	Alive	Not applicable
6	17	M	ALL	Allogeneic	Myeloablative	Tacrolimus, mini-MTX	-153	Coagulopathy	Convalescent plasma, monoclonal antibody, remdesivir, steroids, azithromycin	No	No	No	No	No	Yes	No	Alive	Not applicable
7	6	F	Immune disorder	Allogeneic	RIC	PTCY, Tacrolimus, MMF	-163	Pyrexia, respiratory, GI	None	No	No	Yes	Yes	Yes	No	Yes	Alive	Not applicable
8	16	M	AML	Allogeneic	Myeloablative	PTCY, Tacrolimus, MMF	-96	Pyrexia, respiratory	Remdesivir, steroids	No	No	No	No	No	No	No	Alive	Not applicable
9	15	F	Myelodysplastic syndrome	Allogeneic	Myeloablative	Yes	-97	Pyrexia, respiratory, GI, headaches	Convalescent plasma, remdesivir, steroids, Anakinra	Yes	Yes	Yes	No	No	Yes	No	Deceased	Multi-system organ failure secondary to either protracted initial or secondary COVID-19 infection
10	2	M	AML	Allogeneic	Myeloablative	Tacrolimus, mini-MTX, Abatacept	-73	Pyrexia, respiratory, GI	None	No	No	No	No	No	No	No	Alive	Not applicable

10.3389/fonc.2022.1000215

and eculizumab have demonstrated improved short- and long-term outcomes without significant adverse effects among patients with severe SARS-Cov-2 infection (31, 32).

Currently, the impact of SARS-CoV-2 infection on success of HCT engraftment and ultimate outcome is unknown. Key endothelial cell populations are thought to be involved in homing, engraftment, and restoration of bone marrow functioning following HCT (33, 34). Damage to crucial endothelial cells, such as by recent COVID-19 illness, may conceivably impair success of HCT engraftment. Further investigation into long term transplant outcomes amongst patients with recent SARS-CoV-2 infection who undergo HCT is imperative to understand this relationship.

The following limitations must be considered while interpreting the results of this study. First, although this study was conducted across four pediatric HCT centers, the frequency of patients infected with COVID-19 in the peri-HCT period was low. The sample size of our population of interest is small, which limits study power and ability to perform complete statistical analyses, including multivariate analysis. We strongly encourage ongoing investigation regarding this topic, as more patients will be available for study as the COVID-19 pandemic progresses. Second, he patients included in this research contracted COVID-19 prior to the emergence of the Omicron variants. The Omicron variant infected significantly more pediatric patients nationwide, however severe clinical outcomes were fewer as compared to pediatric patients infected with the Delta variant (35). Follow up studies are recommended to capture the impact of the Omicron strain, as well as future variants that may arise. Third, data regarding patient serologic status and evidence of prior COVID-19 infection was not available for our patient cohort, as these patients were studied prior to the widespread availability of serologic COVID-19 testing. Therefore, we do not have insight into whether these patients experienced a primary or subsequent COVID-19 infection and therefore cannot comment on the impact of subsequent infections on risk of developing endothelial complications. Fourth, we acknowledge a major potential confounder in our underlying HCT variables; patients who contracted COVID-19 prior to HCT were more likely to receive haploidentical grafts, as compared to patients who contracted COVID-19 following HCT. Increased degree of alloreactivity, as well as differences in conditioning and immunosuppression that coincide with haploidentical transplantation, are known risk factors for endothelial complications following HCT (36). Similar studies with larger cohorts are warranted to more effectively control for confounders.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Mayo Clinic IRB, UCSF IRB, MD Anderson Cancer Center IRB, Dana Farber/Boston Children's IRB. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

SA, MK, KMM, MZ, LL designed the research. SA, DR, SK, GC, RB, GM, AF, MK collected the data. SA, KM, MK analyzed the data. SA, MK wrote the paper. All authors provided edits for the paper. SA and MK directly accessed and verified the underlying data reported in the manuscript. All authors should confirm that they had full access to all the data in the study and accept responsibility to submit for publication. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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frontiers | Frontiers in Oncology

TYPE Case Report
PUBLISHED 23 February 2023
DOI 10.3389/fonc.2023.999810

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

RECEIVED 21 July 2022
ACCEPTED 07 February 2023
PUBLISHED 23 February 2023

CITATION
Cervi C, Sápi Z, Bedics G, Zajta E, Hegyi L, Pápay J, Dezső K, Varga E, Mudra K, Bödör C and Csóka M (2023) Case report: Complete and durable response to larotrectinib (TRK inhibitor) in an infant diagnosed with angiosarcoma harbouring a *KHDRBS1-NTRK3* fusion gene. *Front. Oncol.* 13:999810. doi: 10.3389/fonc.2023.999810

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Case report: Complete and durable response to larotrectinib (TRK inhibitor) in an infant diagnosed with angiosarcoma harbouring a *KHDRBS1-NTRK3* fusion gene

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Significant improvements in the survival rates of paediatric cancer have been achieved over the past decade owing to recent advances in therapeutic and diagnostic strategies. However, disease progression and relapse remain a major challenge for the clinical management of paediatric angiosarcoma. Comprehensive genomic profiling of these rare tumours using high-throughput sequencing technologies may improve patient stratification and identify actionable biomarkers for therapeutic intervention. Here, we describe the clinical, histopathological, immunohistochemical and molecular profile of a novel and precision medicine-informed case where a *KHDRBS1-NTRK3* fusion determined by next-generation sequencing-based comprehensive genomic profiling led to complete and sustained remission (clinical and radiological response) in an otherwise incurable disease. Our patient represents the first paediatric angiosarcoma harbouring a targetable *NTRK3* fusion in the literature and demonstrates the first example of targeting this alteration in angiosarcoma using larotrectinib, an *NTRK* inhibitor. Clinical and radiological remission was achieved in under two months of therapy, and the patient is currently in complete remission, 4 month after stopping larotrectinib therapy, which was given over 17 months with only mild side effects reported. Therefore, this remarkable case exemplifies the true essence of precision-based care by incorporating conventional pathology with the why, when, and how to test for rare oncogenic drivers and agnostic biomarkers in paediatric angiosarcoma.

KEYWORDS

precision oncology, next-generation sequencing (NGS), comprehensive genomic profiling (CGP), paediatric soft tissue sarcoma, angiosarcoma, neurotrophic tyrosine receptor kinase (NTRK), *NTRK* fusion gene, larotrectinib

Introduction

Soft tissue sarcomas are a heterogeneous group of malignant tumours derived from mesenchymal cells, accounting for approximately 8-10% of all paediatric malignancies, 0.3% of which are angiosarcomas (1–3). Paediatric angiosarcomas are highly aggressive malignant neoplasms associated with a poor prognosis due to their lack of response to conventional therapy. Due to the heterogeneous genetic profile of primary angiosarcoma, collaborative studies such as the Angiosarcoma Project play an integral role in categorising and applying novel and targeted therapies in this tumour entity (4). However, this project focuses on adult angiosarcoma cases, and little is known about molecular profiling and the application of targeted therapy in paediatric angiosarcoma. The tropomyosin receptor kinase (Trk) family consists of TrkA, TrKB and TrKC transmembrane receptors which are encoded by *NTRK1*, *NTRK2* and *NTRK3* genes, respectively (5). *NTRK* gene fusions are agnostic biomarkers associated with several adult and paediatric solid tumours. In 2018, the FDA approved small-molecule inhibitors of the TRK kinases due to their efficacy regardless of tumour entity, patient age and performance status (6–9). Moreover, due to lowered cost and routine application of next-generation sequencing (NGS) testing in the clinical setting, our knowledge of tumours that harbour *NTRK* fusions, targetable with TRK inhibitors are expanding, improving our understanding of rare tumours and enabling precision-based therapeutic management of clinically challenging tumours (10–12). Here, we describe the first case of paediatric angiosarcoma harbouring a targetable *NTRK3* fusion in the literature and demonstrate the first example of targeting this alteration in paediatric angiosarcoma.

Case report

Clinical presentation

An eight-month-old girl presented to her family practitioner with life-threatening respiratory symptoms. One week later, she was admitted to ICU with hypoxemia, and tachydyspnoea, caused by bilateral pleural effusion and consequent dystelectasis of the lung area. At that time, bilateral chest tubes were performed, and 200 ml of fluid was drained from both sides. A significant amount of pleural effusion appeared every 2-3 days, which required drainage several times. MRI (22/01/21) confirmed the presence of an infiltrating lesion surrounding the trachea, pharynx, superior mediastinum, pleura, chest wall and bilateral pleural effusion. Histological, cytological and immunohistochemical examinations were performed on a neck soft tissue biopsy and pleural fluid aspiration samples, confirming the diagnosis of paediatric angiosarcoma, morphological grade 2. At the time point of diagnosis, therapeutic strategies were limited. From a clinical standpoint, the patient presented in a critical condition and no curative option was available since local therapy, on account of the disease's extent and the child's age. Based on the conventional pathological diagnosis and due to the aggressive behaviour of the tumour, the Cooperative Weichteilsarkom Studiengruppe (CWS)-2012 chemotherapy protocol was applied between 04/02/21 and 08/04/21; considering the patient's age, paclitaxel was initially excluded from the regimen. She received 2 courses of vincristine-adriamycin-cyclophosphamide (VAC). However, due to her clinical condition and the increasing amount of fluid, 4 cycles of paclitaxel were promptly initiated based on the decision of the tumour board. Only a moderate clinical and radiological response was observed to the paclitaxel therapy, with the patient permanently requiring drainage and intensive care.

Cytological and histopathological examinations

Pleural effusion specimen: dissociated atypical tumour cells sometimes arranged in small clusters were observed. Immunocytochemistry showed Vimentin and ERG positivity while WT1 and CAMTA1 were negative. INI1 (SMARCB1) was retained. All these were suggestive of angiosarcoma (Figure 1). **Histology** (biopsy from neck/pleural region): anastomosing vascular spaces lined by atypical flattened endothelial cells with endothelial multilayering were observed infiltrating fat and striated muscle. Immunohistochemistry showed strong ERG nuclear positivity and CD31 cytoplasmic positivity (Figure 1). Cytokeratin (AE1-AE3) and CD34 were negative and no WT1 and CAMTA1 nuclear positivity was observed. Ki-67 proliferation index was 20%. INI1 (SMARCB1) was retained ruling out the possibility of rhabdoid tumour (one of the most common paediatric soft tissue malignancies of this age). The lack of characteristic intracytoplasmic vacuoles and CAMTA1 nuclear immunopositivity ruled out epithelioid haemangioendothelioma. The diagnosis of angiosarcoma (grade 2) was established.

Comprehensive genomic profiling

Comprehensive genomic profiling from DNA and RNA samples extracted from the formalin-fixed paraffin-embedded specimen was performed using the Illumina TruSight Oncology 500 assay on a NextSeq2000 (Illumina) next-generation sequencing platform, in accordance with the manufacturer's instructions. Small insertion/deletions (InDel) or single nucleotide variants (SNV) were detected in the *BRCA1* (c.3700_3704delGTAAA, p.V1234fs*8) and *RNF43* (c.1976delG, p.G659fs*41) genes, respectively. Low-level (<5-fold) amplifications were detected in the *ALK, RET, FGF3, FGF6, EGFR, FGF4, BRCA2*, and *MYCL* genes. An in frame *KHDRBS1-NTRK3* gene fusion, previously unreported in angiosarcoma, was detected, offering therapeutic intervention with a TRK inhibitor (Figure 2). This fusion was validated by fluorescence *in situ* hybridisation (Figure 2).

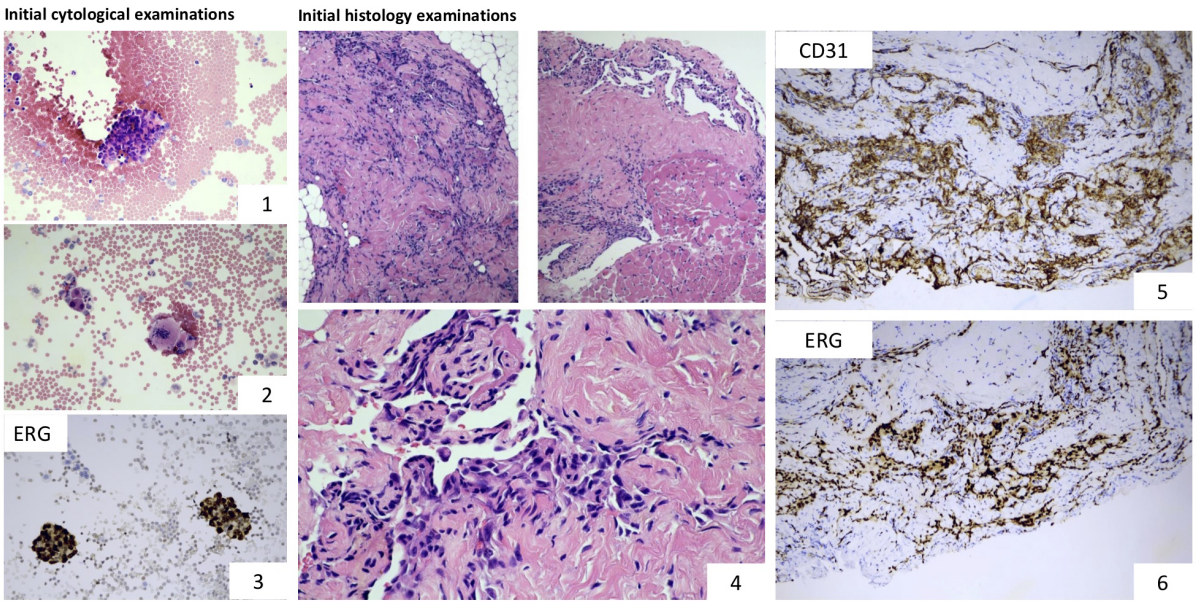

FIGURE 1
Microscopic examinations: Cytological and immunocytochemical examinations performed on the aspirated pleural fluid show the presence of tumour cells (images 1 and 2) and ERG nuclear positivity (image 3). Characteristic histological features of angiosarcoma: proliferating atypical tumour cells forming anastomosing vascular channels. The lining endothelial cells display marked atypia (image 4). Tumour cells showed strong cytoplasmic CD31 and nuclear ERG positivity (images 5 and 6). Based on the morphology and the immunophenotype, the lesion corresponded with the diagnosis of angiosarcoma, grade 2.

Molecularly targeted therapy and clinical follow-up

Based on the *KHDRBS1-NTRK3* fusion, on-label use of larotrectinib (VITRAKVI) (per os 100 mg/m2 twice daily, commencing on 21/04/21) was initiatied. The patient was diligently monitored using clinical, neurological, and radiological assessments. The clinical response to larotrectinib therapy was evident during routine follow-up examinations. At the time of presentation, the patient scored a low Karnofsky performance status score of 20, requiring frequent drainage of her extensive bilateral pleural effusion. Notably, the patient's Karnofsky score significantly improved throughout her treatment trajectory; Karnofsky scores of 40 (following chemotherapy) and 90 (within one week of starting larotrectinib), where she was discharged home without the need for further drainage. To date, the patient has undergone 17 months of larotrectinib therapy, and her current performance status is 100 (mild side effects, normal development, average height, and weight gain were reported). The only noted adverse side effects to larotrectinib were observed in May 2022; grade 2 liver toxicity. Following the CTCAE version 4.03 protocol, the drug dosage was reduced, and the liver toxicity was resolved. On the 12th of July 2022, the patient received the full dosage again. The patient completed her Larotrectinib treatment on the 19th of September 2022 and is still in complete remission (January 2023). Overall, a remarkable and sustainable clinical and radiological response to larotrectinib has been achieved. (Figure 3).

Discussion

Paediatric angiosarcoma is exceedingly rare and aggressive in behaviour compared to cases diagnosed in adulthood. The CWS-2012 international protocol is clear regarding the treatment (chemotherapy, local surgery and or radiotherapy), diagnostic steps, the staging classification, and the follow-up regimen (13). In this case, local therapy was excluded due to the tumour's extent and location; only a moderate response to chemotherapy was achieved using the CWS-2012 protocol. Thus, genomic profiling was performed to identify actionable variants and expand therapeutic possibilities. In light of the paucity of genomic profiling or the application of targeted therapy in paediatric angiosarcoma, we present an unprecedented challenging case and demonstrate a durable clinical response to TRK inhibitor (larotrectinib) based upon a precision oncology approach using next-generation sequencing-based comprehensive genomic profiling.

In normal physiology, neurotrophins are TRK ligands, where ligand binding plays an integral role in several key functions; proliferation and survival of neuronal cells, maintenance of the central and peripheral nervous system, and regulation of behaviour, sensation, movement, and cognition (14–16). *NTRK* gene fusions are the most common mechanism of TRK oncogenic activation found in both adult and paediatric cancers. As a rule of thumb, the frequency of *NTRK* fusions in paediatric oncology is common among rare tumours and rare among common tumours. However, despite the availability of TRK inhibitor therapy owing

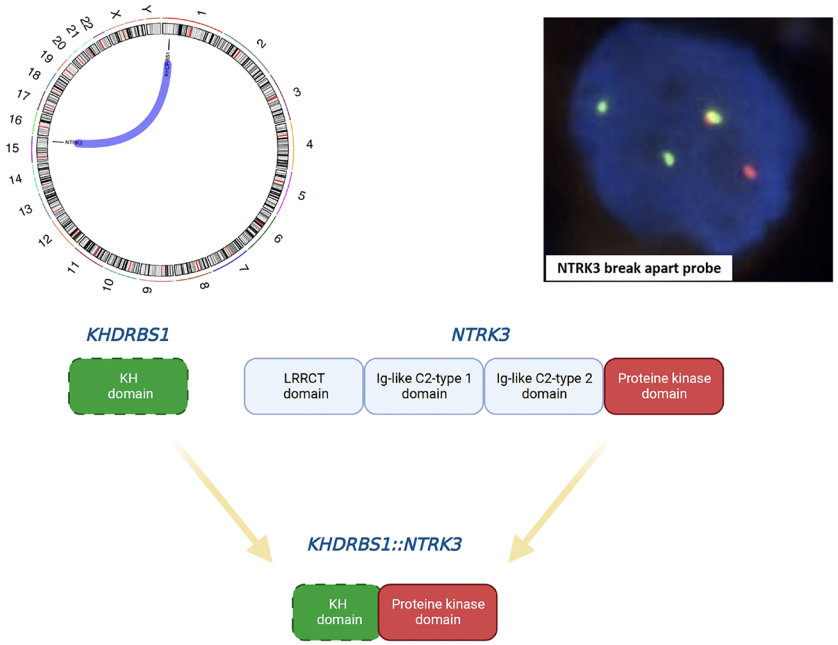

FIGURE 2
Schematic illustration of the in-frame *KHDRBS1-NTRK3* fusion (Circos plot and schematic diagram were created using MAFTOOLS and Biorender, respectively) and confirmation using the *NTRK3* break apart FISH probe. The following breakpoints were identified using the FusionCatcher software: chr1:32039573A and chr15:88033045 for *KHDRBS1* and *NTRK3*, respectively.

to FDA approval in 2018, no data has been published on the application of TRK inhibitor therapy in the management of paediatric angiosarcoma. Additionally, the clinical success of TRK inhibitors, the long-term side effects and the permanent response to these inhibitors, particularly in infants and children, are limited to case reports (17). Fortunately, secondary resistance has only been reported in a minority of patients treated with larotrectinib (6, 18). Clinical trials in large cohorts demonstrate the marked and durable

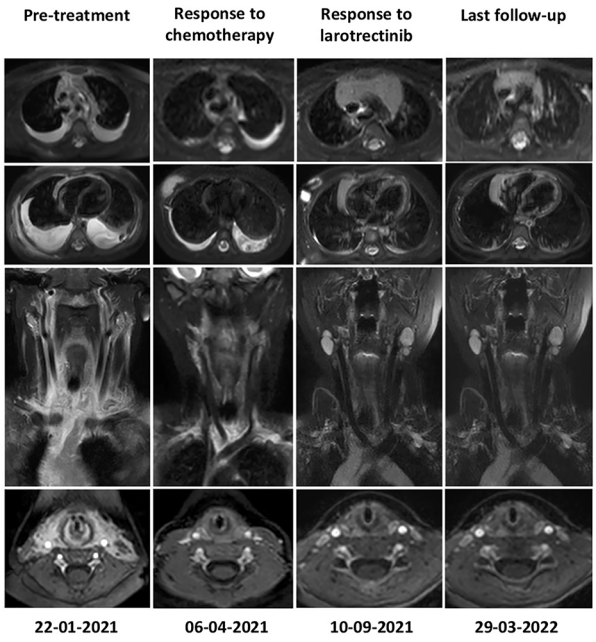

FIGURE 3
Radiological response as determined by Magnetic Resonance imaging before treatment (22.01.21), following administration of the CWS 2012 protocol (06.04.21) and NTRK inhibitor therapy (Larotrectinib) (10.09.21), respectively. Follow-up MRI is performed every three months; the latest images show a sustained response to Larotrectinib therapy as no visible tumour mass is detectable (29.03.22). While a limited therapeutic response upon completion of chemotherapy, a remarkable and sustained complete remission from Larotrectinib was observed.

anti-tumour activity of larotrectinib in adult and paediatric tumours harbouring *NTRK* fusions (locally advanced or metastatic solid tumours regardless of patient age, performance status or tumour entity). Recent evidence suggests that larotrectinib is well-tolerated in adults and children, with predominantly grade 1-2 adverse effects (19). A growing body of studies demonstrates the prevalence and spectrum of *NTRK* fusion partner genes in paediatric soft tissue sarcoma. A previous case report detailed the finding of a *KHDRBS1-NTRK3* rearrangement found in a congenital CD34+ skin tumour. However, TRK inhibition therapy was not provided based on this finding (20). Overall, it is noticeable that different frequencies are shown across these various studies, most likely due to differences in available diagnoses and referral bias (21, 22). Considering these studies, a paradigm shift towards genetic profiling of rare tumours is paramount to identify clinically targetable biomarkers, improve patient stratification and expand therapeutic options in these clinically challenging tumours.

This case study demonstrates the first application of TRK inhibitors in managing paediatric angiosarcoma. Since there are no guidelines regarding the duration of therapy or follow-up regimens of paediatric patients undergoing TRK inhibitor therapy, further large-scale trials are required. Ongoing specific digital-droplet PCR assays coupled with close clinical and radiological assessments will enable continuous monitoring of the measurable residual disease from serial liquid biopsy specimens to assess the depth of the achieved remission and detect relapse or resistance mutations.

Conclusion

Paediatric angiosarcoma is a rare subtype of soft tissue sarcoma associated with a poor prognosis and limited therapeutic options. To our knowledge, we present the first case of precision-based management of paediatric angiosarcoma harbouring a *KHDRBS1-NTRK3* fusion gene that responded to an *NTRK* inhibition. The routine application of next-generation sequencing of rare paediatric malignancies associated with poor prognosis expands the morphological spectrum and clinical relevance of targeted therapies, especially in cases that do not respond to conventional therapy. Furthermore, the true essence of precision-based care by incorporating conventional pathology with the why, when, and how to test for oncogenic drivers and agnostic biomarkers in rare soft tissue sarcomas is demonstrated throughout this unprecedented case.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by Regional, Institutional Scientific and Research Ethics Committee, Semmelweis University. Written informed consent was obtained from the individual(s) and minor(s)' legal guardian/next of kin to publish any potentially identifiable images or data in this article.

Author contributions

CC, CB and MC wrote the manuscript. EZ, CC, LH and GB performed sequencing experiments and analyzed data. ZS, JP, KD, EV, KM and MC contributed with pathological analysis and participated in the clinical management of the patient. All authors contributed to the article and approved the submitted version.

Funding

The study was funded by the EU's Horizon 2020 research and innovation program under grant agreement No. 739593, and Elixir Hungary.

Conflict of interest

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

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frontiers | Frontiers in Pediatrics

TYPE Original Research
PUBLISHED 08 March 2023
DOI 10.3389/fped.2023.1091735

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Pediatrics

RECEIVED 07 November 2022
ACCEPTED 20 February 2023
PUBLISHED 08 March 2023

CITATION
Wang H, Wang L, An S, Ma Q, Tu Y, Shang N and Pan Y (2023) American college of radiology ovarian-adnexal reporting and data system ultrasound (O-RADS): Diagnostic performance and inter-reviewer agreement for ovarian masses in children.
Front. Pediatr. 11:1091735.
doi: 10.3389/fped.2023.1091735

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American college of radiology ovarian-adnexal reporting and data system ultrasound (O-RADS): Diagnostic performance and inter-reviewer agreement for ovarian masses in children

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Objective: To evaluate the diagnostic performance and inter-observer agreement of the American College of Radiology Ovarian-Adnexal Reporting and Data System Ultrasound (O-RADS) in the diagnosis of ovarian masses in children.
Methods: From June 2012 to December 2021, 163 ovarian masses in 159 patients with pathologic results were retrospectively analyzed. Each mass was classified into an O-RADS category according to the criteria. The diagnostic performance of O-RADS for detecting malignant ovarian masses was assessed using histopathology as the reference standard. Kappa (*k*) statistic was used to assess inter-observer agreement between a less-experienced and a well-experienced radiologist.
Results: Out of 163 ovarian masses, 18 (11.0%) were malignant and 145 (89.0%) were benign. The malignancy rates of O-RADS 5, O-RADS 4, and O-RADS 3 masses were 72.7%, 34.6%, and 4.8%, respectively. The area under the receiver operating characteristic curve was 0.944 (95% CI, 0.908–0.981). The optimal cutoff value for predicting malignant ovarian masses was >O-RADS 3 with a sensitivity, specificity, and accuracy of 94.4%, 86.2% and 86.2% respectively. The inter-observer agreement of the O-RADS category was good (*k* = 0.777).
Conclusions: O-RADS has a high diagnostic performance for children with ovarian masses. It provides an effective malignant risk classification for ovarian masses in children, which shows high consistency between radiologists with different levels of experience.

KEYWORDS

O-RADS, ovarian masses, ultrasound, diagnostic performance, children

Introduction

Ovarian mass is a common disease in women, but its incidence in children is approximately 2.2–2.6 per 100,000 (1). The pathological type of ovarian mass in children is complex, most of which are benign masses, while malignant tumors account for approximately 4%–22% (1). Different from adult ovarian tumors that are mainly epithelial tumors, pediatric ovarian tumors are mainly derived from germ cell tumors. Most

Abbreviations
O-RADS, Ovarian-Adnexal Reporting and Data System Ultrasound; *k*, kappa; PACS, Picture Archiving and Communication System; ROC, Receiver operating characteristic; AUC, area under the curve.

pediatric ovarian tumors (including malignant tumors) have a relatively good prognosis if treated timely, and the 5–10-year survival rates can reach 80%–90% (2). However, with the improvement of survival rate, the requirement of fertility preservation is a key consideration that poses a challenge to the choice of surgical method for ovarian masses in children. In this setting, surgeons need to balance the need for fertility preservation with that that of accurate staging and evaluation of the resection range of malignant tumors (3, 4). Moreover, avoiding resection of ovaries with benign tumors reduces the risk of premature menopause and its short and long-term sequelae such as infertility, osteoporosis, cardiovascular disease, and neurocognitive effects (5, 6). Preoperative assessment of the risk of malignancy for ovarian masses is a key imperative in order to strike a balance between fertility preservation and more aggressive cancer treatment (7, 8). Use of ultrasound for the differential diagnosis of benign and malignant ovarian masses in children is mainly based on the size and physical properties of the masses, but there are obvious limitations (8–10).

Structured reporting of the ultrasound findings of ovarian masses was identified by a consensus working group of a Society of Radiologists in Ultrasound as a key step for improving the management of women with ovarian masses (11). The structured reporting systems mainly include ovarian-adnexal reporting and data system (O-RADS), gynecologic imaging reporting and data system (GI-RADS), International Ovarian Tumor Analysis (IOTA) "Simple Rules" and "ADNEX" models. These models have shown a high diagnostic performance for women with ovarian masses (12–17). However, application of these models to pediatric ovarian tumors has not been reported. Therefore, the purpose of this study was to evaluate the diagnostic performance of ultrasound O-RADS in the differential diagnosis of benign and malignant ovarian tumors in children, so as to identify a more objective and standardized method for the preoperative evaluation of ovarian tumors in children.

Materials and methods

Population

We retrospectively analyzed children with ovarian masses confirmed by histopathological examination of surgical specimens at the Guangdong Women and Children Hospital between June 2012 and December 2021. Data pertaining to demographic characteristics, clinical examinations, pathologic diagnosis, surgical findings, and follow-up data were retrieved from the electronic medical case records. Inclusion criteria were: (a) age <18 years; (b) ovarian mass was detected by ultrasonography, and surgical treatment was performed to obtain clear pathological results; (c) ultrasound images were complete and clear. Exclusion criterion: histological findings were obtained more than 120 days after the ultrasound examination. Finally, 159 children were enrolled in this study.

Examination methods

Ultrasound examinations were performed using high-resolution color Doppler ultrasound diagnostic apparatus such as Samsung (WS80A, RS80A), Aloka (α10), GE (VOLUSON E8, VOLUSON E6), Hitachi (HIVISON Preirus, 60/70), and Mindray (DC-8, Kunlun 7). The frequency of convex array probe was 2–8 MHz, the frequency of linear array probe was 4–12 MHz, and the frequency of intracavity probe was 5–10 MHz. Routine abdominal examination was performed. The bladder was moderately filled before examination. Patients were placed in a supine position to fully expose the lower abdomen, and the pelvic and abdominal cavity (if necessary) were comprehensively scanned. The size of the uterus and bilateral ovaries, and presence of any ovarian or pelvic mass was recorded. The size, shape, boundary, relationship with surrounding tissues, internal echo and blood flow of the tumor were recorded. When necessary, trans-rectal ultrasound examination was also performed for differential diagnosis. Written informed consent was obtained from a parent or guardian and the examination was performed in the presence of a parent or guardian. The imaging data of all cases were stored in Picture Archiving and Communication System (PACS) for analysis. All patients were followed up after surgery, and the results were confirmed by histopathological examination of surgical specimens.

Retrospective images analysis

Ultrasound images were retrieved from the PACS. Before study set up, a resident radiologist with 3 years of experience learned the theory of the O-RADS lexicon and Risk Stratification and Management System. O-RADS classification of ultrasound images was performed by the resident radiologist, who was blinded to the clinical information and pathologic results. The radiologist described the ultrasound features and assigned an O-RADS category for each mass.

The O-RADS categories are (18): O-RADS 0: incomplete evaluation; O-RADS 1: definitively benign. Normal ovaries; O-RADS 2: almost certainly benign category (<1% risk of malignancy); O-RADS 3: low-risk category (1% to <10% risk of malignancy); O-RADS 4 intermediate-risk category (10% to <50% risk of malignancy); O-RADS 5: high-risk category (>50% risk of malignancy).

To assess inter-observer agreement with respect to O-RADS categorization between radiologists with different levels of experience, another radiologist with 9 years of experience performed a separate analysis for all the masses. The radiologist described the ultrasound features and performed O-RADS classification of the masses.

Reference standard

The reference standard was histological diagnosis based on surgical specimen. Histopathology of masses were classified by

the World Health Organization International Classification of Ovarian Tumors (19). As the same surgical intervention is recommended for borderline and malignant ovarian masses, borderline masses were defined as malignant (20).

Statistical analysis

Data analyses were performed using SPSS version 20.0. Categorical variables were compared using the Chi-squared test. Non-normally distributed continuous variables were presented as median and inter-quartile range, and between-group differences were assessed using Mann–Whitney *U* test. Receiver operating characteristic (ROC) curve analysis was performed to calculate the areas under the curve (AUC) and determine the optimal cut-off values. Two-tailed *P*-values <0.05 were considered indicative of statistical significance.

We used Kappa (*k*) statistics to assess inter-observer agreement of ultrasound features and O-RADS category. The *k* values were interpreted as follows: poor agreement = 0.01–0.20; fair agreement = 0.21–0.40; moderate agreement = 0.41–0.60; good agreement = 0.61–0.80; very good agreement = 0.81–1.0.

Results

Patients and ovarian masses

A total of 163 ovarian masses in 159 patients were included in this study. The median age of patients was 13.0 (3.0, 16.0) years (range, 0–17). The detailed age distribution is shown in Table 1. There were 4 bilateral ovarian masses (all benign) and 155 unilateral ovarian masses. 145 (89.0%) masses were benign and 18 (11.0%) masses were malignant proven by pathology. Benign masses were mainly mature teratoma ($N = 78$, 53.8%), while malignant masses were mainly germ cell tumors ($N = 9$, 50.0%). The median age of the malignant group was 14.0 (11.8, 16.0) years, and that of the benign group was 13.0 (2.5, 15.0) years. The difference was not statistically significant ($P = 0.052$). The maximum median diameter of the tumor in the malignant group was 11.5 (7.3, 13.4) cm, which was significantly higher than that in the benign group [6.5 (4.9, 9.4) cm; $P = 0.012$].

TABLE 1 Age distribution of the patients.

Age (year)	Numbers(%)
<1	30 (18.4)
1–3	12 (7.4)
4–6	6 (3.7)
7–9	5 (3.1)
10–12	25 (15.3)
13–15	42 (25.8)
16–17	43 (26.4)

Ultrasound features

The ultrasonic characteristics of benign and malignant ovarian tumors are compared (χ^2 test) in Table 2. There was a significant difference between masses categorized as benign and malignant with respect to maximum diameter of masses, external contour, color score, and ascites ($P < 0.05$), which are the key terms in the O-RADS ultrasound lexicon.

O-RADS classification

A total of 163 masses were assessed. Of the 105 ovarian masses categorized as O-RADS 2, none was malignant; of the 21 ovarian masses categorized as O-RADS 3, one ovarian mass was malignant; of the 26 ovarian masses categorized as O-RADS 4, nine ovarian masses were malignant; and of the 11 ovarian masses categorized as O-RADS 5, eight were malignant. Table 3 summarizes the O-RADS classification and histological diagnosis of the ovarian masses.

Diagnostic performance

The malignancy rates of O-RADS 5, O-RADS 4 and O-RADS 3 lesions were 72.7%, 34.6%, and 4.8% respectively. For O-RADS classification, the area under the ROC curve was 0.944 (95% CI, 0.908–0.981) and the optimal cutoff value for predicting malignant ovarian masses was > O-RADS 3 (Figure 1). The sensitivity, specificity, accuracy, positive predictive value, and negative

TABLE 2 Ultrasound characteristics of ovarian masses.

Ultrasonic characteristics		Final diagnosis		χ^2 test
		Benign ($n = 145$)	Malignant ($n = 18$)	*P*-value
Lesion category	Unilocular, no solid component	40	0	<0.001
	Unilocular cyst with solid component (s)	45	1	
	Multilocular cyst, no solid elements	22	0	
	Multilocular cyst with solid component (s)	35	10	
	Solid or solid appearing	3	7	
Maximum diameters of lesions (D)	D < 10 cm	114	7	<0.001
	D ≥ 10 cm	31	11	
Irregular external contour	Yes	5	6	<0.001
	No	140	12	
Color score	1	113	3	<0.001
	2	25	7	
	3	3	5	
	4	4	3	
Ascites	Yes	9	4	0.018
	No	136	14	

TABLE 3 O-RADS classification and histological diagnosis of 163 ovarian masses.

Histologic diagnosis	O-RADS 2	O-RADS 3	O-RADS 4	O-RADS 5	Total
Benign adnexal masses	105	20	17	3	145
Mature teratoma	60	11	6	1	78
Follicular cyst	25	2	2	0	29
Serous cystadenoma	4	0	1	0	5
Mucinous cystadenoma	13	6	5	0	24
Corpus luteum	2	0	0	0	2
Endometrioma	0	0	1	0	1
Ovarian Theca-fibroma	0	0	1	2	3
Other benign adnexal masses	1	1	1	0	3
Malignant adnexal masses	0	1	9	8	18
Germ cell tumor	0	0	3	6	9
Sex cord-stromal tumor	0	1	3	2	6
Borderline tumor	0	0	3	0	3
Total	105	21	26	11	163

predictive value of O-RADS 4 and 5 categorization for malignant lesions were 94.4%, 86.2%, 86.2%, 45.9%, and 99.2%, respectively.

The O-RADS 4 lesions include the following: (1) unilocular cyst with solid component; (2) multilocular cyst, no solid elements; (3) multilocular cyst with solid component; (4) solid or solid appearing. If unilocular cyst with solid component and multilocular cyst with no solid elements are categorized as O-RADS 4A masses and the remaining cystic lesions with solid components are categorized as O-RADS 4B masses, the malignancy rates were 10.0% and 50.0%, respectively (Table 4),

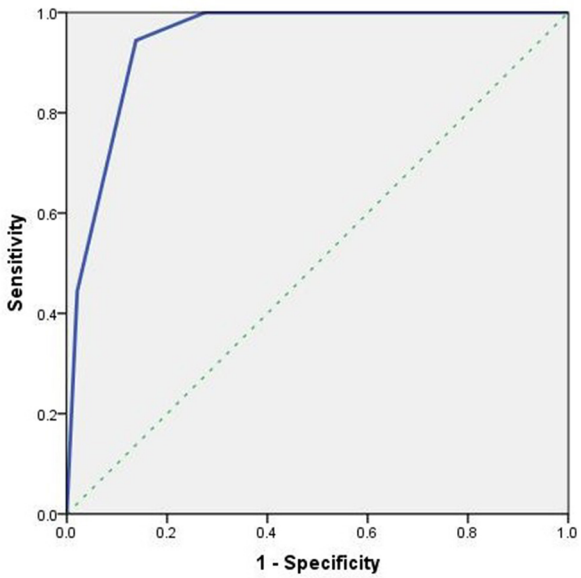

FIGURE 1
Receiver operating characteristic (ROC) curve analysis of O-RADS.

TABLE 4 Malignant risk in sub-groups of O-RADS 4 masses.

	O-RADS 4A	O-RADS 4B	
Benign	9	8	17
Malignant	1	8	9
Total	10	16	26

which indicated significant improvement in risk stratification ($P = 0.037$).

Figures 2–5 show the ultrasound findings of O-RADS 2, 3, 4 and 5 masses.

Inter-observer agreement between different levels radiologists

Inter-observer agreement between a radiologist with 3 years of experience (Observer 1) and a radiologist with 9 years of experience (Observer 2) was assessed regarding ultrasound features and O-RADS category. The inter-observer agreement of the O-RADS category was good ($k = 0.777$, $P < 0.001$) (Table 5). With respect to description of ultrasound features, we found very good inter-observer agreement respect to with identification of ascites ($k = 0.853$, $P < 0.001$) and classification of masses categories ($k = 0.847$, $P < 0.001$). The inter-observer agreement was good for color scores ($k = 0.655$, $P < 0.001$) and external contour ($k = 0.681$, $P < 0.001$).

Discussion

In this study we evaluated the diagnostic performance and inter-observer agreement with respect to ACR O-RADS categorization of ovarian masses in a Chinese pediatric cohort. In addition, we verified the ultrasound risk stratification of O-RADS classification, and evaluated the differences between benign and malignant ovarian tumors with respect to the key terms in the dictionary. The results showed that the diagnostic performance of O-RADS for children with ovarian masses was good, and the inter-observer reliability among radiologists with different levels of experience was high. Our findings also suggest that O-RADS provide an effective risk stratification of malignant tumors for children ovarian masses, and the sub-classification of O-RADS4 masses can provide better risk stratification.

In our cohort, malignant tumors accounted for 11.0% of ovarian tumors which is consistent with a multi-center study by Madenci et al. (21). We also found that the maximum diameter of malignant tumors was significantly larger than that of benign tumors, which is consistent with that reported by Papic et al. and Lala et al. (10, 22). In 2020, ACR officially released a consensus guide for ultrasound risk stratification and management for O-RADS (18). The consensus guide is based on the O-RADS ultrasound dictionary published by the ACR ultrasound working group in 2018 (23). It is the only dictionary and risk-stratification system that contains all risk categories and related

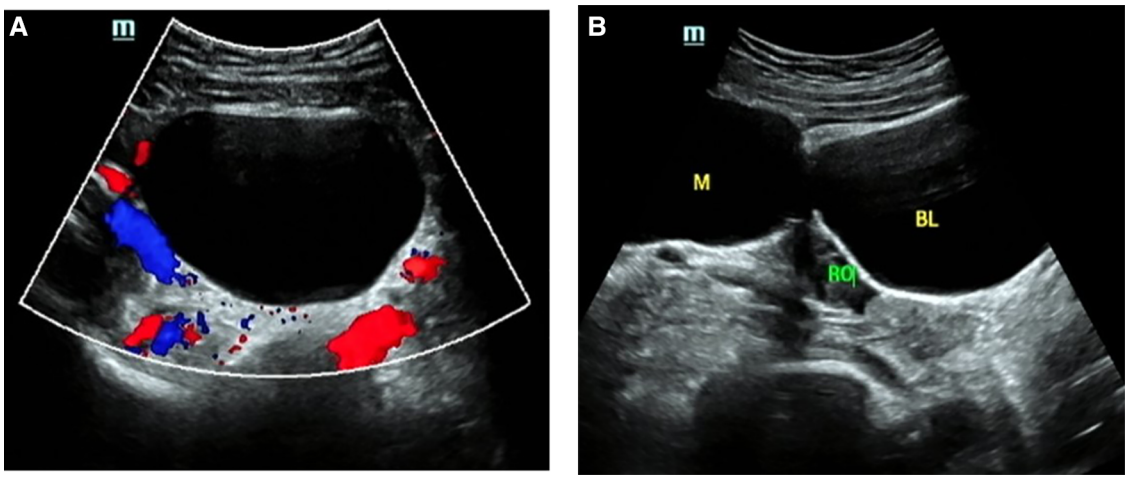

FIGURE 2
O-RADS 2, maximum diameters of lesion 79 mm, pathology: mature teratoma. (**A**) Unilocular cyst, no solid elements, color score 1; (**B**) The mass was located beside the right ovary. M, mass; RO, right ovary; BL, bladder.

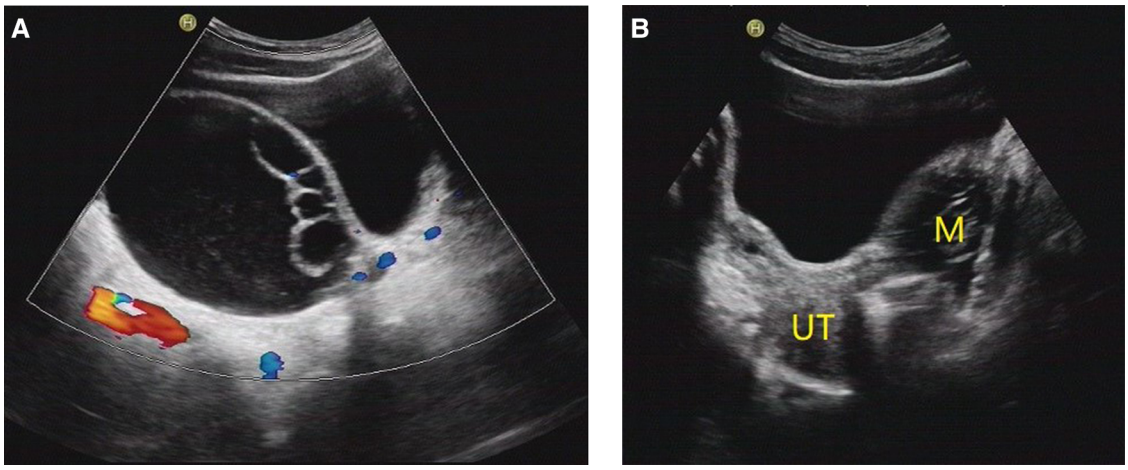

FIGURE 3
O-RADS 3, maximum diameters of lesion 93 mm, pathology: serous cystadenoma. (**A**) Multilocular cyst, no solid elements, color score 2; (**B**) The mass was located on the left side of the uterus. M, mass; UT, uterus.

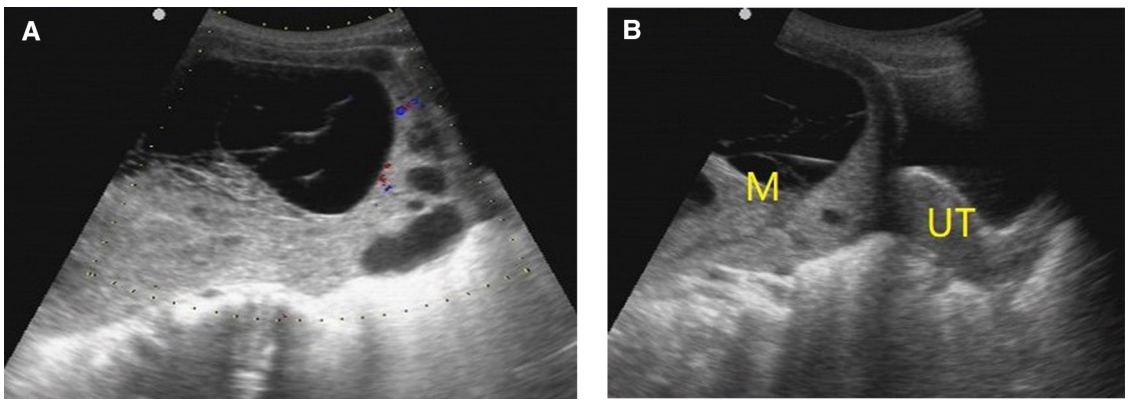

FIGURE 4
O-RADS 4, pathology: yolk sac tumor. (**A**) Multilocular cyst, solid elements, color score 2; (**B**) The mass was located above the uterus. M, mass; UT, uterus.

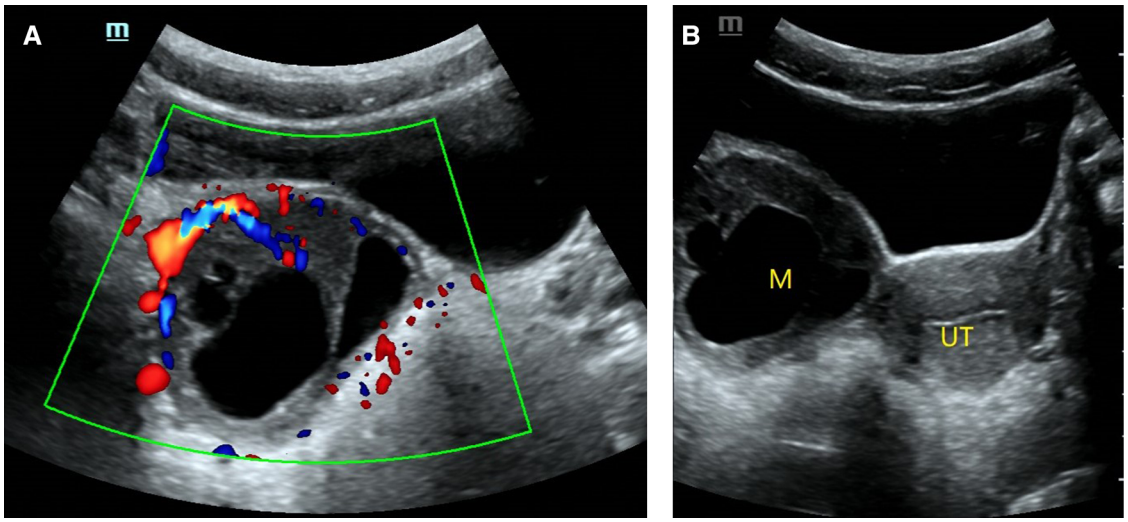

FIGURE 5
O-RADS 5, pathology: moderate differentiated Sertoli-leydig cell tumor. (A) Multilocular cyst, solid elements, color score 3; (B) The mass was located on the right side of the uterus. M, mass; UT, uterus.

TABLE 5 Inter-observer agreement of O-RADS classification.

		Observer 2				Total
		O-RADS 2	O-RADS 3	O-RADS 4	O-RADS 5	
Observer 1	O-RADS 2	92	9	3	1	105
	O-RADS 3	1	17	3	0	21
	O-RADS 4	0	0	22	4	26
	O-RADS 5	0	0	0	11	11
Total		93	26	28	16	163

management schemes. Studies have shown that ultrasound O-RADS has good value in differentiating benign from malignant ovarian tumors in adult women (24).

Therefore, this study expounded the application value of O-RADS classification for pediatric ovarian masses from the aspects of inter-observer consistency, diagnostic threshold, and diagnostic performance, so as to provide an objective, reliable, and standardized classification method for the identification of benign and malignant ovarian masses in children.

In this study, we observed a significant difference benign and malignant tumors with respect to color Doppler score, presence of ascites, lesion type, lesion size, and external contour ($P < 0.05$), which are also the key terms in the O-RADS ultrasound lexicon. However, we note that not all terms in the lexicon are selected into the risk stratification system, such as acoustic shadowing. We found that 4 of 18 malignant masses had acoustic shadows (22.2%), and 46 of 145 benign masses had acoustic shadows (31.7%). Acoustic shadow may be a key feature to distinguish between benign and malignant tumors.

In our cohort, the number of cases with O-RADS1 class was 0, because all masses in our study were confirmed by surgery and pathological results, and O-RADS1 class indicates normal adnexa, which was not included in this study. The number of O-RADS 2 was the largest, because of the large proportion of benign masses in this group, and it was also consistent with the distribution of disease. In this study, the malignant rates of O-RADS in categories 2, 3, 4 and 5 were 0, 4.8%, 34.6% and 72.7%, respectively. Based on pathological results, the malignant rate of each O-RADS category was basically consistent with the risk recommended by the system (18). Cao L et al. also found a similar risk of malignancy for O-RADS 2 (0.45%), 3 (1.10%), 4 (34.46%), and 5 (89.57%) masses in adult patients with adnexal masses (24). Another study also showed a similar risk (2.8%) of malignancy for O-RADS 3 masses (25). The recommended risk of O-RADS 4 is between 10% and 50%, and in this study the malignant risk of O-RADS 4 was 34.6%. Therefore, it is still difficult to determine whether O-RADS 4 masses are malignant or benign. We tried to subdivide O-RADS 4 masses into two categories to obtain more accurate stratification. O-RADS 4A was associated with a malignant risk of 10%; For O-RADS 4B, the risk of malignancy was 50%. Therefore, the sub-classification of O-RADS4 masses can provide better risk stratification ($P < 0.05$). Therefore, we believe that it is very important to sub-classify O-RADS4.

In this study, the diagnostic threshold of ultrasound O-RADS classification for the differential diagnosis of benign and malignant ovarian masses in children was > O-RADS 3, which was consistent with the diagnostic threshold of O-RADS in the differential diagnosis of benign and malignant ovarian tumors in adults (24, 26). In this study, O-RADS 4–5 were diagnosed as malignant masses. The diagnostic performance of O-RADS classification for benign and malignant masses was very high (AUC: 0.944), indicating that O-RADS provides a good tool for differentiating benign and malignant ovarian masses in children, with high sensitivity (94.4%) and negative predictive value (99.2%). At the same time, the specificity of O-RADS classification for detecting malignant tumors in this study was 86.2%, and the positive predictive value was 45.9%, indicating that a large proportion of tumors diagnosed as malignant by O-RADS classification were benign tumors, which was mainly due to the fact that the ultrasonographic images of benign tumors such as mature teratoma, benign cystadenoma, and follicular membrane-fibroma may be characterized by multilocular tumors accompanied by malignant signs such as solid component, echo clutter, solid tumors, and slightly rich blood flow signals.Therefore, benign tumors are also likely to be classified into O-RADS 4–5 categories. For this subset of children, further differential diagnosis should be made based on clinical manifestations, laboratory tests (21, 27), MRI (28), and other imaging examinations.

It is very important to study the consistency of O-RADS classification results among different radiologists because O-RADS classification is based on ultrasound features which are liable to be influenced by subjectivity. Cao et al. (24) found good consistency between inexperienced radiologists and expert radiologists with respect to the description and classification of accessory lesions. This indicates that O-RADS has a good application for radiologists with different levels of experience. Pi et al. (26) reported that, even without specialized training, experienced ultrasound readers can achieve excellent diagnostic results and higher inter-reader reliability through self-study of guidelines and cases. So does O-RADS have good classification consistency in assessing the risk of malignant ovarian masses in children? This study found good consistency between radiologist with different experience levels with respect to O-RADS classification of pediatric ovarian masses ($k = 0.777$). Our results showed that the results of O-RADS classification may not rely on the work experience of ultrasound doctors, and to some extent, it reduces the diagnostic differences caused by subjective factors, and facilitates the communication between radiologists and clinicians. Our findings suggest that O-RADS classification is an objective classification method for the evaluation of ovarian masses in children, which is worthy of popularization and application.

However, this was a retrospective study of ultrasound images, which may have introduced an element of bias. Due to the low incidence and low malignant rate of ovarian masses in children, this study is based on a low number of tumors (163 benign and 18 malignant). In addition, in this retrospective study, it was not possible to identify the indications for surgery in patients with O-RADS 2 or 3 lesions. O-RADS also recommends close follow-up or management by gynecological experts for O-RADS 2 and O-RADS 3 masses. However, there are some limitations for O-RADS: Unlike the IOTA ADNEX model, O-RADS cannot provide individual risk of each lesion and is more cumbersome; And it needs to be emphasized that in this study the O-RADS is not a screening test but is used to attempt differentiating between benign and malignant tumors, once these tumors have been observed by ultrasound; O-RADS provides recommendations purely based on findings and often suggests unnecessary prolonged follow-up or additional testing; O-RADS may not be suitable for experts who always perform well, if not just checking images.

Conclusions

In this study, O-RADS showed a high diagnostic performance for children with ovarian masses. Its high sensitivity and negative predictive value may help avoid missed diagnosis of ovarian malignant tumors in children, and provide the basis for timely intervention and preoperative evaluation. It provides an effective malignant risk classification for ovarian masses in children, which shows high consistency between radiologists with different levels of experience. In particular, this study found that the sub-classification of O-RADS4 masses can provide better risk stratification. Therefore, prospective, multicenter studies are required to provide more robust evidence of the diagnostic performance of O-RADS for pediatric masses.

Data availability statement

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

Ethics statement

The Hospital Ethics Committee approved this retrospective study. The requirement of obtaining written informed consent of the patients was waived off.

Author contributions

HW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing - original draft; LW: Conceptualization, Formal analysis, Investigation, Methodology, Resources; SA: Validation, Visualization, Investigation; QM: Validation, Visualization, Investigation; YT: Investigation, Methodology; NS: Funding acquisition, Project administration, Resources, Writing - review & editing; YP: Methodology, Funding acquisition, Project administration, Resources, Writing - review &

editing. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by Guangdong Province Key field Research and Development Plan Project (Nos.:2020B1111130002)

Acknowledgments

The authors thank the Department of Ultrasound of Guangdong Women and Children Hospital for examine the children.

Conflict of Interest

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

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