ADVERSE AND TOXIC EFFECTS OF CHILDHOOD CANCER TREATMENTS

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ADVERSE AND TOXIC EFFECTS OF CHILDHOOD CANCER TREATMENTS

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Table of Contents

05 ***Editorial: Adverse and Toxic Effects of Childhood Cancer Treatments***
Antonio Ruggiero, Roderick Skinner and Wael Zekri Khaled Zekri

08 ***Treatment-Related Toxicities During Anti-GD2 Immunotherapy in High-Risk Neuroblastoma Patients***
Thomas Blom, Roosmarijn Lurvink, Leonie Aleven, Maarten Mensink, Tom Wolfs, Miranda Dierselhuis, Natasha van Eijkelenburg, Kathelijne Kraal, Max van Noesel, Martine van Grotel and Godelieve Tytgat

18 ***Influence of Methylenetetrahydrofolate Reductase C677T and A1298C Polymorphism on High-Dose Methotrexate-Related Toxicities in Pediatric Non-Hodgkin Lymphoma Patients***
Suying Lu, Xiaoqin Zhu, Wei Li, Huimou Chen, Dalei Zhou, Zijun Zhen, Feifei Sun, Junting Huang, Jia Zhu, Juan Wang, Yizhuo Zhang and Xiaofei Sun

27 ***Fertility Preservation in Female Pediatric Patients With Cancer: A Clinical and Regulatory Issue***
Serena Brancati, Lucia Gozzo, Laura Longo, Daniela Cristina Vitale, Giovanna Russo and Filippo Drago

38 ***Dasatinib in the Management of Pediatric Patients With Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia***
Claudio Cerchione, Franco Locatelli and Giovanni Martinelli

45 ***Management of Oral Mucositis in Children With Malignant Solid Tumors***
Giorgio Attinà, Alberto Romano, Palma Maurizi, Sara D'Amuri, Stefano Mastrangelo, Michele Antonio Capozza, Silvia Triarico and Antonio Ruggiero

52 ***Use of Chronic Prescription Medications and Prevalence of Polypharmacy in Survivors of Childhood Cancer***
Celeste L. Y. Ewig, Yi Man Cheng, Hoi Shan Li, Jasper Chak Ling Wong, Alex Hong Yu Cho, Freddie Man Hong Poon, Chi Kong Li and Yin Ting Cheung

63 ***Neurocognitive and Behavioral Outcomes of Chinese Survivors of Childhood Lymphoblastic Leukemia***
Liwen Peng, Lok Sum Yang, Perri Yam, Chun Sing Lam, Agnes Sui-yin Chan, Chi Kong Li and Yin Ting Cheung

76 ***Cardiac Surveillance for Early Detection of Late Subclinical Cardiac Dysfunction in Childhood Cancer Survivors After Anthracycline Therapy***
Rosaria Sofia, Veronica Melita, Antonio De Vita, Antonio Ruggiero, Alberto Romano, Giorgio Attinà, Lisa Birritella, Priscilla Lamendola, Antonella Lombardo, Gaetano Antonio Lanza and Angelica Bibiana Delogu

85 ***Digital Phenotyping and Dynamic Monitoring of Adolescents Treated for Cancer to Guide Intervention: Embracing a New Era***
Johanna M. C. Blom, Chiara Colliva, Cristina Benatti, Fabio Tascedda and Luca Pani

91 ***Cisplatin Ototoxicity: Examination of the Impact of Dosing, Infusion Times, and Schedules In Pediatric Cancer Patients***

Miranda L. Camet, Anne Spence, Susan S. Hayashi, Ningying Wu, Jennifer Henry, Kara Sauerburger and Robert J. Hayashi

99 ***A Prospective Study on Fertility Preservation in Prepubertal and Adolescent Girls Undergoing Hematological Stem Cell Transplantation***

Ida Wikander, Frida E. Lundberg, Hanna Nilsson, Birgit Borgström and Kenny A. Rodriguez-Wallberg

107 ***Follicle Rescue From Prepubertal Ovaries After Recent Treatment With Cyclophosphamide—An Experimental Culture System Using Mice to Achieve Mature Oocytes for Fertility Preservation***

Xia Hao, Amandine Anastácio, Laia Viñals-Ribé, Ana Santamaria Lacuesta, Christina Diakaki, Sara Alonso de Mena, Kui Liu and Kenny A. Rodriguez-Wallberg

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EDITORIAL
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Editorial: Adverse and Toxic Effects of Childhood Cancer Treatments

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Editorial on the Research Topic

Adverse and Toxic Effects of Childhood Cancer Treatments

The development of effective treatments for cancer has been a landmark achievement of medical research in the modern era. Now, many children with cancer can be cured with dramatic increases in the survival rates of most types of childhood cancers over the last 30 years (1). Most antineoplastic drugs can produce acute toxicities (i.e., nausea, vomiting, mucositis, alopecia,…) which are generally reversible once the chemotherapy is completed (2–4). Some anticancer drugs are associated with delayed toxic effects which can became evident even many years from the chemotherapy (5, 6). Therefore, as the number of survivors from childhood cancers is increasing, late effects are now becoming major concerns highlighting the importance of long-term follow-up (7, 8).

This Research Topic includes 12 manuscripts focusing on the pathophysiology, diagnosis, treatment, and outcomes of acute and long-term side effects in children treated for cancer.

In their retrospective study, Lu et al. aimed to investigate the relationships between the methylenetetrahydrofolate reductase (MTHFR) C677T/A1298C and high-dose methotrexate (HD-MTX)-related toxicities in a homogenous group of children with non-Hodgkin lymphoma (NHL). Patients harboring mutant C677T genotype were more vulnerable to oral mucositis, leucopenia, and thrombocytopenia while those with mutant A1298C genotype were more likely to develop anemia and leucopenia but less susceptible to vomiting. These results can be of great interest for clinicians as predictors of MTX toxicity can help to determine the appropriate individual dosage minimizing adverse effects.

Attinà et al. reported their experience of the management of oral mucositis as well as documenting its risk factors. Mucositis was a common complication of treatment for childhood malignancies with 50% of patients suffering from at least one episode. Its occurrence was related to the presence of neutropenia, number of chemotherapy courses, and type of tumor. The WHO oral mucositis scale appeared to be a valuable tool for assessing the severity of mucositis and the relative effectiveness of pain relief with opioids.

The study by Blom T et al. provides a focus on the treatment-related toxicities during immunotherapy with dinutuximab, IL-2, GM-CSF, and isotretinoin in 26 high-risk neuroblastoma patients receiving treatment according to the DCOG NBL2009 protocol. The most common grade ≥3 toxicities were pain, central venous catheter-related infections, and fever.

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In total, 310 grade ≥3 toxicities were recorded in 124 courses and a higher number of toxicity episodes was observed in children receiving IL-2.

With a broad perspective on its potential for cancer treatment, the review by Cerchione et al. outlines the role of Dasatinib in the management of childhood Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL). The use of the second generation tyrosine kinase inhibitors (TKIs), such as Dasatinib, has significantly improved the outcome for pediatric patients with Ph+ALL when used in combination with the standard chemotherapy protocols. The role of Dasatinib in combinations with other molecules for new risk-adapted/MRD-driven clinical trials in relapsed Ph+ALL.or in a post hemopoietic stem cell transplantation setting has to be investigated in future studies.

Several articles within this Research Topic explore recent developments in fertility preservation. Brancati et al. underline the variations in access to available preventive measures. Pharmacological treatment with GnRHa has become an alternative non-invasive and well-tolerated approach, especially in those who cannot access cryopreservation options due to clinical and/or logistic issues. Nevertheless, at present, only very limited evidence from randomized clinical trials on the use of GnRHa in adolescents with cancer is available. In their translational research model, Hao et al. explore the feasibility of follicle isolation and culture from prepubertal mice ovaries recently treated with cyclophosphamide as an alternative fertility preservative method. In addition, the reproductive potential of these follicles regarding the final achievement of mature competent oocytes has been also studied. The follicles obtained were able to grow, and had spindle and chromosome configurations that were normal in the mature oocytes. Wikander et al. report their prospective study involving prepubertal and adolescent girls undergoing HSCT and the outcomes of fertility preservation treatments performed before or after HSCT. Although, the study has some limitations due to the small size, authors were able to demonstrate that fertility preservation can be achieved before and also after HSCT so enhancing the chances of future pregnancies.

Camet et al. assessed the effect of dosing, infusion times, and schedules of cisplatin administration on hearing loss in children receiving cisplatin. Their findings show that the amount of cisplatin infused per dose was strongly associated with an increased risk of hearing loss as well as cumulative cisplatin dose and young age at treatment.

Evidence is accumulating that for childhood cancer survivors, late effects including cardiotoxicity, chronic health conditions, neurocognitive and behavioral functioning can have a negative impact on their quality of life. Blom JM et al. suggest the adoption of digital phenotyping and dynamic monitoring for adolescents treated for cancer. Their integrated multi-dimensional model, as part of a digital toolbox, can represent a new approach for the development of age-appropriate resources that could help them in managing their disease and the treatment side effects. The work of Sofia et al. aimed at detecting late subclinical cardiac dysfunction in children treated for cancer with anthracyclines. The early recognition of such sub clinical cardiac dysfunction may be fundamental for facilitating prompt appropriate medical management. They did not identify statistically significant correlations between echocardiographic parameters (2D, strain and 3D assessment) and age at cancer diagnosis or duration of follow-up. Significantly reduced 3D left ventricle ejection fraction was reported in children treated with anthracyclines despite no significant differences in 2D ejection fraction and longitudinal strain values.

Ewig et al. conducted a retrospective study on chronic polypharmacy prescription among childhood cancer survivors. They confirm the potentially high incidence of late, and often permanent, health complications arising from intensive treatment with combination chemotherapy and ionizing radiation especially for children with central nervous system tumors or survivors who undergone hematopoietic stem cell transplantation. For physicians who are dedicated to taking care of these patients, future work requires appropriate consideration for this vulnerable population.

In their prospective study on Chinese survivors of childhood acute lymphoblastic leukemia (ALL), Peng et al. detected that most young survivors had normal cognitive and behavioral function during the early phase of survivorship although subjects with chronic health conditions or negative socio-environmental were at higher risk of executive cognitive dysfunction.

Although the studies presented in this Research Topic cannot comprehensively cover the whole breadth of this topic, the result of each individual study opens new research and clinical questions helpful to build a new health system model aimed at reducing the risk of major adverse and toxic effects related to cancer treatment.

AUTHOR CONTRIBUTIONS

AR conceived the draft. RS and WK contributed to the manuscript text and editing. RS supervised the manuscript writing process. All authors provided critical feedback and helped shape the direction of the manuscript.

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ORIGINAL RESEARCH
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Treatment-Related Toxicities During Anti-GD2 Immunotherapy in High-Risk Neuroblastoma Patients

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The introduction of immunotherapy using an anti-GD2 antibody (dinutuximab, ch14.18) has significantly improved survival rates for high-risk neuroblastoma patients. However, this improvement in survival is accompanied by a substantial immunotherapy-related toxicity burden. The primary objective of this study was to describe treatment-related toxicities during immunotherapy with dinutuximab, IL-2, GM-CSF, and isotretinoin. A retrospective, single center analysis of immunotherapy-related toxicities was performed in twenty-six consecutive high-risk neuroblastoma patients who received immunotherapy as maintenance therapy in the Princess Máxima Center (Utrecht, Netherlands). Toxicities were recorded and graded according to the CTCAE. Particular attention was drawn to pain and fever management and toxicities leading to dose modifications of dinutuximab and IL-2. Twenty-three patients (88%) completed all six courses of immunotherapy. Disease progression, isotretinoin-associated liver toxicity, and catheter-related infection in combination with peripheral neuropathy were reasons for immunotherapy discontinuation. The most common grade ≥3 toxicities for courses 1–5, respectively, were pain, catheter-related infections, and fever. In total, 310 grade ≥3 toxicities were recorded in 124 courses. Thirty-three grade 4 toxicities in 19/26 patients and no grade 5 toxicities (death) were seen. Fifty-nine percent of grade ≥3 toxicities were recorded in the two courses with IL-2. Catheter-related bloodstream infections were identified in 81% of patients. Four of these episodes led to intensive care admission followed by full recovery (grade 4).

Keywords: neuroblastoma, immunotherapy, dinutuximab, ch14.18, anti-GD2 antibody, safety, toxicity

HIGHLIGHTS

Immunotherapy-related toxicities after induction and consolidation according to the Dutch Childhood Oncology Group (DCOG) NBL2009 treatment protocol are considerable but manageable. More toxicity is observed in the immunotherapy courses containing IL-2.

INTRODUCTION

Despite intensive treatment regimens, patients with high-risk neuroblastoma experienced poor survival outcomes (1). The introduction of immunotherapy using a chimeric anti-GD2 monoclonal antibody (dinutuximab, ch14.18) combined with immunostimulatory cytokines [interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF)] has significantly improved survival rates for these patients (2). The disialoganglioside GD2, has relative tumor-selective expression, with only weak expression in normal human tissues like neurons, melanocytes, and peripheral nerve pain fibers (3), making it an attractive target for neuroblastoma-specific immunotherapy. However, as early as in the first clinical reports, the substantial toxicity burden caused by the ch14.18 antibody was recognized (4, 5), with patients suffering from intense, morphine-responsive pain, intermittent fever, allergic reactions (exanthema, urticaria), and changes in blood pressure. To increase antibody-dependent cellular cytotoxicity (ADCC) and the subsequent antitumor effect, the addition of immunostimulatory cytokines (IL-2, GM-CSF) was studied with encouraging results (6, 7). Here, the clinical benefit has to be weighed against the potential toxicity of these cytokines. Treatment-related toxic effects have resulted in treatment discontinuation and even deaths (2, 8, 9).

From 2016 onwards, high-risk neuroblastoma patients receive immunotherapy as maintenance therapy in the Princess Máxima Center for Pediatric Oncology (Utrecht, The Netherlands). The aim of this study was to describe treatment-related toxicities from immunotherapy with dinutuximab, cytokines IL-2 and GM-CSF, and isotretinoin in a cohort of 26 high-risk neuroblastoma patients treated with induction and consolidation therapy according to the DCOG (Dutch Childhood Oncology Group) NBL2009 high-risk group protocol (10). Specifically, we studied pain and fever management and treatment-related toxicities leading to dose modifications of dinutuximab and IL-2.

Additionally, we performed a non-systematic literature review on toxicity associated with ch14.18 antibody-based immunotherapy in patients with neuroblastoma.

MATERIALS AND METHODS

Patient Population

A retrospective, single center analysis of immunotherapy-related toxicities was performed in twenty-six consecutive high-risk neuroblastoma patients who received dinutuximab-based immunotherapy as maintenance therapy between August 2016 and October 2019 in the Princess Máxima Center for Pediatric Oncology (Utrecht, The Netherlands). The high-risk patient cohort consisted of International Neuroblastoma Risk Group [INRG (11)] stage M and ≥ 12 months at diagnosis, or INRG stage L2 with *MYCN* amplification. All patients had completed induction and consolidation therapy according to the DCOG NBL2009 treatment protocol (10), which is based on the standard arm of the German GPOH (Gesellschaft für Pädiatrische Onkologie und Hämatologie) NB2004 high-risk protocol (12). Patients who achieved at least partial response were eligible to receive immunotherapy. Patients with relapse were not included. Other requirements were Lansky Performance Scale score of ≥60%, adequate organ functions, and full recovery from any toxicities from previous treatments.

Immunotherapy Protocol

An overview of the six immunotherapy courses is provided in **Supplementary Figure 1**. The first five patients received dinutuximab (ch14.18/SP2/0; United Therapeutics Corporation, USA) under a named-patient program at a dose of 17.5 mg/m^2 per day as a 10 h (20 h maximum) intravenous infusion on 4 consecutive days. After the approval of dinutuximab beta by the European Medicines Agency (EMA) in May 2017, patients received dinutuximab beta (ch14.18/CHO; EUSA Pharma, Netherlands) at a dose of 20 mg/m^2 per day as an 8 h (16 h maximum) infusion on 5 consecutive days. During courses 1, 3, and 5, GM-CSF was administered for 14 consecutive days. During courses 2 and 4, IL-2 was administered by continuous intravenous infusion at a dose of 3.0 x 10^6 and 4.5 x 10^6 IU/m^2/day in weeks 1 and 2, respectively. All patients received isotretinoin at a dose of 160 mg/m^2 per day for 14 days per course. Course 6 solely consisted of isotretinoin.

Pain Management and Prophylactic Medication

Pain management consisted of oral gabapentin (15 mg/kg/day in three doses) starting 7 days prior to start of dinutuximab infusion, and intravenous acetaminophen (60 mg/kg/day in four doses, with a maximum of 4 g/day) and morphine (10 μg/kg/h) starting 1 and 2 h before the start of dinutuximab infusion, respectively. Gabapentin and morphine were continued during dinutuximab infusion. Individual patients were closely monitored by the pain anesthesiologist. In case of inadequate pain control, a personalized combination of intermittent IV morphine boluses, esketamine (0.1–0.4 mg/kg/h), clonidine (1–6 μg/kg/day), and amitriptyline (0.5–2 mg/kg/day) was used. When morphine was not tolerated due to side effects or renal failure, piritramide was used instead.

Prophylactic treatment for immune-related symptoms with antihistamines consisted of the combination of clemastine, cetirizine, and ranitidine.

Toxicity

Vital parameters, laboratory results including blood culture results, and other toxicities were prospectively recorded in

Abbreviations: GD2, Disialoganglioside 2; IL-2, Interleukin-2; GM-CSF, Granulocyte-macrophage colony-stimulating factor; ADCC, Antibody-dependent cellular cytotoxicity; COG, Children's Oncology Group; DCOG, Dutch Childhood Oncology Group; INRG, International Neuroblastoma Risk Group; GPOH, Gesellschaft für Pädiatrische Onkologie und Hämatologie; IV, intravenous; CTCAE, Common Terminology Criteria for Adverse Events; WB-FPRS, Wong–Baker Faces Pain Rating Scale; VAS, Visual analogue scale; CRI, Catheter-related infection; PICU, Pediatric intensive care unit; SPSS, Statistical Package for the Social Sciences; ALT, Alanine transaminase; AST, Aspartate transaminase; LTI, long-term infusion; EMA, European Medicines Agency; CVAD, central venous access device; CLABSI, central line-associated bloodstream infection.

patients' medical and nursing files and retrospectively graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE; version 3.0). In case toxicities were not listed in CTCAE version 3.0 (e.g. Cytokine release syndrome), CTCAE version 5.0 was used. Data from grade 1–2 toxicities are not reported, with the exception of fever and grade 1–2 toxicities that resulted in dose modifications of dinutuximab and/or IL-2.

During dinutuximab infusion, pain scores were obtained at least every 4 h. Intensity of pain was assessed using the COMFORT Behavior Scale for patients <3 years of age (13), the Wong–Baker Faces Pain Rating Scale (WB-FPRS) for patients between the age of 3 and 8 (14), and the visual analogue scale (VAS) for children ≥8 years of age (15). COMFORT scores >24 and WB-FPRS and VAS scores ≥7 were considered as severe pain (grade 3). In the case of disabling pain, pain was graded as grade 4.

The maximum body temperature per day was used to assess fever instances. Acetaminophen and diclofenac were, respectively, used as first- and second-line pharmacologic antipyretic therapy. We defined catheter-related infection (CRI) as blood culture-proven bacteremia in association with (1) clinical evidence of infection (fever, tachycardia, hypotension, etc.), (2) no probable other site of infection or cause for bacteremia, and (3) treated with systemic antibiotic therapy. Pediatric intensive care unit (PICU) admission due to a CRI was graded as grade 4. Empirical antibiotic therapy started after fever and discontinued after normalization of symptoms in combination with negative or contaminated blood cultures were not regarded as infections. For this study, blood culture results were reviewed by a pediatric infectious disease physician and categorized based on identification and pathogenicity of isolated bacteria.

Dose Modifications of Dinutuximab and Interleukin-2

We collected all dose modifications of dinutuximab and IL-2 from medical and nursing files. We differentiated between a 50% decrease in the infusion rate, temporary interruption and complete cessation of dinutuximab/IL-2 infusion. For every dose reduction, the causative toxicity was recorded. To investigate the effect of dinutuximab dose modifications, we calculated the total administered dose per course per patient as percentage of intended dose.

Statistical Analysis

McNemar's test for paired data was used to compare the incidence of toxicities between courses containing GM-CSF (courses 1, 3, and 5) and IL-2 (courses 2 and 4). An independent sample t-test was used for antibody type, sex, vital status, myeloablative conditioning regimen, and number of grade ≥3 toxicities. Pearson correlation for association was used for age and number of grade ≥3 toxicities. P values <.05 were considered statistically significant. All statistical analysis was performed using SPSS v.25.0 (IBM, USA).

Literature Review

A literature search was conducted in PubMed/MEDLINE (January 1980–March 2020) to identify reports addressing toxicity of ch14.18 antibody-based immunotherapy in children treated for neuroblastoma. The search strategy and selection criteria can be found in **Supplementary Table 1**. To identify rare complications, case reports were included in this non-systematic review.

RESULTS

Literature Review

To evaluate the toxicity associated with ch14.18 antibody-based immunotherapy in patients with neuroblastoma, we performed a review of the existing literature and identified six studies reporting grade ≥3 toxicities (2, 8, 9, 16–18). The most prevalent reported toxicities in these studies are listed in **Table 1**. Cross-study comparisons should be made with caution, since differences exists between these studies in antibody origin/manufacturer, concomitant cytokines administered, infusion times, and toxicity criteria used.

Overall, the most common grade ≥3 toxicity observed is pain with incidence rates ranging from 16% (without IL-2) to 62% (with IL-2) (8, 9). Pain occurred most frequently during the first immunotherapy course and at lower rates in subsequent courses (2, 8, 9, 16, 18). Grade ≥3 infections are reported in a range of 25%–39% (2, 8, 17), and fever in a range of 9%–67% (9, 18), with more grade ≥3 fever occurring in courses containing IL-2 (9). Seventy-seven percent more infections and 86% more catheter-related infections were seen in the immunotherapy group in comparison with standard isotretinoin therapy (2).

In three studies, grade 5 toxicity (death) was reported with an incidence rate of 1% (2, 8, 9). Causes of death were capillary leak syndrome (2×) (2, 8), in one case after an IL-2 overdose (medication error) (2), sudden cardiac arrest (9), and acute respiratory distress syndrome in the context of an infection (8).

The number of patients that permanently discontinued immunotherapy due to toxicity ranges from 5% to 16% (2, 8, 9, 16, 17). Half of the treatment discontinuations were caused by allergic reactions (9, 16, 17).

Several case reports and series have described rare side effects of ch14.18 antibody-based immunotherapy. Central neurotoxicity ranges in severity from disorientation and confusion (2, 8) to disabling cases of myelitis (19) and encephalopathy (2, 8, 20, 21), which warrant corticosteroid treatment and immediate discontinuation of immunotherapy. Full recovery is most likely to occur, although exceptions have been described (8, 21).

Ocular complications as mydriasis and accommodation deficits are frequently encountered and seldomly severe (8, 9, 22). Ocular symptoms are completely reversible in most patients and discontinuation of immunotherapy does not seem to be warranted (22).

Unusual and severe gastrointestinal complications have been associated with ch14.18 antibody-based immunotherapy in the

TABLE 1 | Literature overview of reported non-hematological immunotherapy-related grade ≥3 toxicities.

Study - Year	Yu - 2010	Marachelian – 2016	Mody – 2017	Ladenstein - 2018	Mueller - 2018	Ozkaynak - 2018
Patients	**n = 137**	**n = 28**	**n = 16[a]**	**n = 406**	**n = 53**	**n = 105**
Immunotherapy composition						
Antibody	Ch14.18/NCI	Ch14.18/UTC[b] Ch14.18/NCI[b]	Dinutuximab	Dinutuximab beta	Dinutuximab beta[c]	Dinutuximab
Cytokines	IL-2 + GM-CSF	IL-2 + GM-CSF	GM-CSF	IL-2 (randomized)[d]	IL-2	IL-2 + GM-CSF
Other	Isotretinoin	Isotretinoin	Temozolomide/ Irinotecan	Isotretinoin	Isotretinoin	Isotretinoin
Toxicity (%)						
Pain	52	33 vs.29	44	16 vs.26[d]	38	22 – 41[e]
Fever	39	48 vs.44	25 (+ infection)	14 vs.40[d]	9	5 – 59[e]
Infection	39	n.r.	n.r.	25 vs.33[d]	n.r.	n.r.
CRI	13	n.r.	n.r.	n.r.	n.r.	n.r.
Hypotension	18	7 vs.11	13	4 vs.17[d]	2	4 – 17[e]
Hypersensitivity	25	n.r.	n.r.	10 vs.20[d]	2	2 – 10 [e] (AR)
Urticaria	13	n.r.	n.r.	5 vs.10	8	n.r.
CLS	23	n.r.	0	4 vs.15[d]	13	0 – 4[e]
Hypokalemia	35	26 vs.26	38	n.r.	n.r.	n.r.
Hyponatremia	23	19 vs.19	19	n.r.	n.r.	n.r.
Increased ALT	23	15 vs.4	6	17 vs.23 [d] (+AST)	n.r.	n.r.
Hypoxia	13	4 vs.11	25	n.r.	6	n.r.
Neurotoxicity						
Central	4	n.r.	n.r.	1.6 vs.5,8[d,f]	n.r.	n.r.
Peripheral	n.r.	n.r.	6	0.5 vs.3.1[d,g]	2	n.r.
Grade 5 toxicity (%)	1 (n = 1)	0	0	1 (n = 2)	0	1 (n = 1)
Antibody dose reductions (%)	n.r.	n.r.	38 (6/16)	n.r.	n.r.	43 (45/104)
Discontinuation IT due to toxicity (%)	15 (16/107)	7 (2/28)	13 (2/16)	5 (9/183) vs.16 (31/188)	n.r.	8 (8/104)

CRI, catheter-related infection; CLS, capillary leak syndrome; ALT, Alanine transaminase; AST, Aspartate transaminase; AR, allergic reaction; IT, immunotherapy; n.r., not reported. [a]Maintenance + relapsed/refractory patients. [b]Randomized crossover study comparing ch14.18-UTC (United Therapeutics Corporation) with ch14.18-NCI (National Cancer Institute). [c]24 h continuous infusion. [d]Comparison of immunotherapy with and without IL-2. [e]Reported as range for courses 1–5. [f]Disorientation/hallucinations, seizures, posterior reversible encephalopathy syndrome, toxic demyelinating encephalopathy + coma. [g]Paresthesia, motor deficits, tetraparesis.

form of necrotizing enterocolitis (23) and small bowel pneumatosis and ischemia (24).

Patient Characteristics

Between 2016 and 2019, a cohort of twenty-six consecutive high-risk neuroblastoma patients (11 girls and 15 boys) were treated with dinutuximab-based immunotherapy and were included in this study. Patient characteristics are summarized in **Table 2**. The median age at diagnosis was 3.5 years (range 4 months–18 years). Most patients were INRG stage M and ≥ 12 months of age at diagnosis (n=22), while some were children with INRG stage L2 with *MYCN* amplification (n=4). The median time between diagnosis and the start of immunotherapy was 10 months (range 8–23 months). Five patients received dinutuximab, 20 patients received dinutuximab beta, and one patient received both antibodies.

Toxicities

Twenty-three patients completed all six courses of immunotherapy. In two patients, immunotherapy was discontinued after two courses. One patient developed unacceptable toxicities: grade 4 catheter-related infection (CRI) in combination with bilateral mydriasis and severe peripheral sensory and motor neuropathy which improved over time but did not resolve completely. In another patient, immunotherapy was discontinued because of disease progression. In a third patient, isotretinoin was permanently discontinued during course 5 due to liver toxicity (grade 4 elevated AST and ALT).

In total, 310 grade ≥3 toxicities were recorded during 124 immunotherapy courses (courses 1–5). Grade ≥3 toxicities were not evenly distributed among the five courses; with most toxicities reported in courses 2 and 4 involving IL-2 (20; 36; 12; 23; 10% for courses 1–5, respectively). All 26 patients experienced at least one grade ≥3 toxicity. Thirty-three grade 4 toxicities in 19/26 patients and no grade 5 toxicities (death) were seen. In **Figure 1**, the most common grade ≥3 toxicities for courses 1–5 are depicted, while all toxicities encountered during the immunotherapy courses are listed in **Supplementary Table 2**. Here only the highest grade per course per patient is given. Pain was the most common grade ≥3 toxicity observed in 96% (25/26) of patients at some point during immunotherapy (courses 1–5). Sixty-five percent (17/26) of patients suffered from disabling, grade 4 pain. Esketamine and clonidine were used in 88% (23/26) and 50% (13/26) of patients, respectively, due to inadequate pain control. Grade ≥3 pain was most frequent during immunotherapy course 1, occurring in 88% of patients. During course 5 the proportion of patients with grade ≥3 pain decreased to 42% (p=.003).

The second and third most common grade ≥3 toxicity were CRIs in 19%, 65%, 25%, 54%, 21%, and fever in 19%, 62%, 13%, 42%, 13% of patients in courses 1–5, respectively. Although both toxicities occurred more frequently in immunotherapy courses

TABLE 2 | Patient characteristics of high-risk neuroblastoma cohort.

Patient characteristics	n = 26 No.	(%)
Sex		
Female	11	42
Male	15	58
Age at diagnosis (months)		
Median	41.5	
Range	4–224	
Age at start immunotherapy (months)		
Median	55	
Range	16–240	
INRG stage at diagnosis[a]		
Stage L2	4	15
Stage M	22	85
Location primary tumor		
Adrenal	19	73
Sympathetic side chain	7	27
***MYCN* status**		
Amplified	11	42
Single copy	15	58
Treatment – Induction		
Standard induction chemotherapy + Surgery	18	69
+ additional (chemo)therapy	8	12
Treatment - Consolidation – Myeloablative conditioning regimen		
Carboplatin/Etoposide/Melphalan (CEM)	9	35
Busulfan/Melphalan (BuMel)	17	65
Treatment – Maintenance - Anti-GD2 antibody		
Dinutuximab	5	19
Dinutuximab beta	20	77
Both antibodies	1	4
Disease status at start immunotherapy[b]		
Complete response (CR)	14	54
Partial response (PR)	12	46
Vital status at end of follow-up		
Alive	20	77
Dead	6	23
Follow-up (End immunotherapy – Last control; months)		
Median	22	
Range	9 – 39	

[a]*As defined by International Neuroblastoma Risk Group (11).* [b]*As defined by International Neuroblastoma Response Criteria (25).*

that contained IL-2 (courses 2 and 4) as compared with courses that contained GM-CSF (courses 1, 3, and 5), a statistically significant difference between IL-2 and GM-CSF courses was only found for catheter-related infections (p=.039), and not for fever (p=.057).

In **Figure 2** and **Supplementary Table 3**, all grade ≥3 toxicities per patient are shown for courses 1–5. Here toxicities may be documented multiple times per course, with a maximum of once per day. In total, 441 grade ≥3 toxicities were recorded for the 23 patients that completed all six courses of immunotherapy, with a median of 18 (range 10–37) grade ≥3 toxicities per patient. No statistically significant difference in the number of grade ≥3 toxicities per patient were noted between the patients that received dinutuximab or dinutuximab beta (p=.754). The same holds true for sex (p=.275), myeloablative conditioning regimen (p=.708), and vital status at the end of follow-up (p=.948). Age at diagnosis (p=.908) and at the start of immunotherapy (p=.925) were not significantly correlated with the number of grade ≥3 toxicities per patient.

Fever Management

All 26 patients experienced fever during immunotherapy; 81% of patients suffered from fever >40.0°C (grade 3). In 124 courses of immunotherapy (courses 1–5), 341 instances of fever were recorded (**Table 3**). Sixty-three percent of fever episodes were recorded in the immunotherapy courses with IL-2 (course 2: 34% and course 4: 29%). During these episodes, 274 blood cultures were taken from which 52 CRIs were identified in 81% of patients. Four of these episodes were life-threatening (grade 4) and led to intensive care admission followed by full recovery. All 26 patients received immunotherapy through a Hickman central venous access device (CVAD). Twelve patients (46%) had a Hickman CVAD implanted shortly before the start of immunotherapy, the other patients (54%) received a Hickman CVAD earlier in their treatment history (i.e., induction or consolidation phase). Surgical removal of the CVAD was performed in 54% (28/52) of CRIs. Ten patients (38%) did not require a CVAD removal during immunotherapy.

Staphylococcus species were identified in 29% (15/52) of CRIs, in 14/15 blood cultures a Staphylococcus aureus was isolated. In 29% (15/52) of CRIs, Gram-negative pathogens were detected. **Supplementary Table 4** lists the identified bacteria isolated in all CRIs.

Dose Modifications

To reduce the toxicity burden of immunotherapy, the infusion rate of dinutuximab may be decreased. In case of more severe toxicity, temporary interruption or permanent discontinuation of infusion may be necessary. In our cohort, the dinutuximab dose was modified in 81% (21/26) of patients (**Table 4**). Forty-two percent (11/26) of patients did not receive 100% of the intended dinutuximab dose at some point during courses 1–5. In courses 1 and 3, all patients received 100% of the intended dinutuximab dose. In course 2, 19% (5/26) did not receive the planned dinutuximab dose. Here, two patients, both suffering from a CRI, only received <50%. In course 4, 21% (5/24) received ≥50 to <100% of cumulative intended dinutuximab dose. Lastly, in course 5, 8% (2/24) did not receive the planned dinutuximab dose. Here, one patient received <50% of the dinutuximab dose after therapy discontinuation due to severe coughing.

Although more patients did not receive 100% of the intended dinutuximab dose in the courses containing IL-2 (n=9) as compared with courses containing GM-CSF (n=2), this difference was not significant (p=.065). Grade ≥3 pain, the most common toxicity overall, led to dose modifications in 5 patients. All five patients, however, received 100% of cumulative intended course dose of dinutuximab.

The treatment of patients with IL-2 had to be modified due to treatment-related toxicities in 54 and 48% of patients in courses 2 and 4, respectively (**Supplementary Table 5**). The most prevalent toxicities preceding IL-2 dose modifications were coughing, CRIs, AST/ALT abnormalities, and fever.

Disease Outcome

At the last follow-up, six patients (23%) had died of disease after the start of immunotherapy. One patient, with a complete

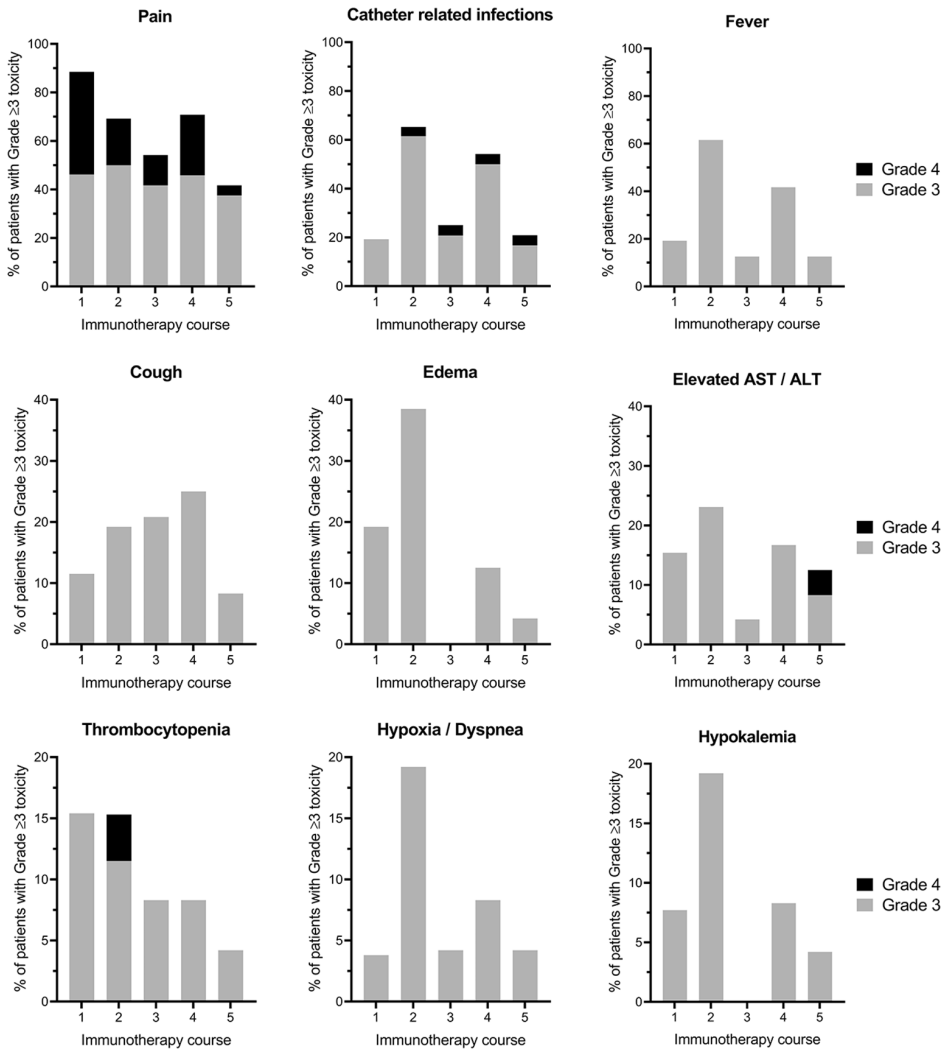

FIGURE 1 | The most prevalent immunotherapy-related grade ≥3 toxicities per course. Proportion of patients experiencing grade ≥3 toxicities per cycle (1–5) of immunotherapy are displayed. The highest grade of toxicity per patient per course is shown. Please note the different scales of the Y-axis. AST, Aspartate transaminase; ALT, Alanine transaminase.

response before the start of immunotherapy, suffered from cerebral metastases and only completed two courses of immunotherapy. The other five patients had a relapse after immunotherapy completion. In four of these patients, skeletal relapses were detected at the response assessment after immunotherapy course 6. In the fifth patient, a mediastinal soft tissue relapse was discovered 8 months after the completion of immunotherapy.

DISCUSSION

Treatment-related toxicity during immunotherapy with dinutuximab, cytokines IL-2 and GM-CSF, and isotretinoin after induction and consolidation according to the DCOG NBL 2009 treatment protocol is substantial. All 26 analyzed patients suffered from grade ≥3 toxicities, 73% suffered from grade 4 toxicities and no grade 5 toxicities (death) were seen. Pain, fever, coughing, edema, and liver enzyme abnormalities were among the most common toxicities observed. These results are in line with earlier reports in which comparable immunotherapy regimens were used (2, 9, 16). A large interpatient variability in grade ≥3 toxicity burden was observed, possibly related to the pharmacokinetic variability of dinutuximab in disposition and clearance in children (26, 27). Generally, immunotherapy-related toxicities were transient and resolved with the discontinuation of antibody and/or cytokine infusion, or with appropriate supportive care (pain/fever management). However, 12% of patients (3/26) could not complete all immunotherapy courses due to toxicity, and in one of these patients the peripheral neuropathy did not resolve completely. Peripheral neurotoxicity is a rare, but severe side effect of immunotherapy with a reported prevalence between 2 and 6% (8, 17, 18).

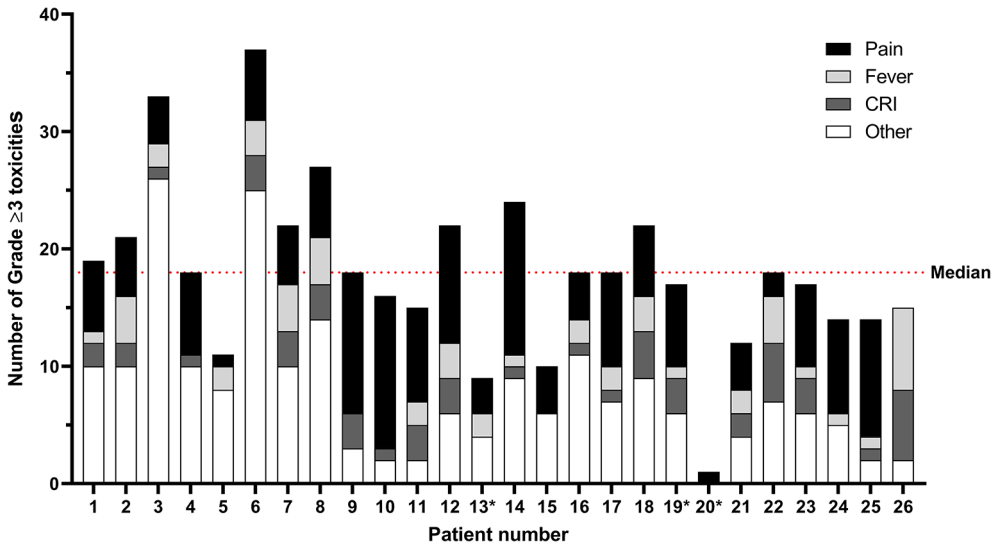

FIGURE 2 | Immunotherapy-related grade ≥ 3 toxicities per patient for courses 1–5. Toxicities may be documented multiple times per course, with a maximum of once per day. The median number of grade ≥3 toxicities per patient is 18 (range 10–37) for the 23 patients who completed all six courses of immunotherapy. The category "Other" comprises toxicities in alphabetical order from the CTCAE categories Allergy; Blood/bone marrow; Constitutional, Cardiac; Gastrointestinal, Infection; Lymphatics; Metabolic/laboratory; Neurology; Ocular/visual; Pulmonary; and Vascular. All individual toxicities encountered are listed in **Supplementary Table 2**. *Patients 13, 19, and 20 did not complete all cycles of immunotherapy. CRI, catheter-related infections.

TABLE 3 | Fever instances, blood cultures, and catheter-related infections.

		Course 1 (n = 26)		Course 2 (n = 26)		Course 3 (n = 24)		Course 4 (n = 24)		Course 5 (n = 24)		Total
		No.	No. patients	No.	No. patients	No.	No. patients	No.	No. patients	No.	No. patients	No.
Fever instances		56	25	115	26	40	20	99	24	31	18	341
Grade (in %)	**1**	41		23		73		28		68		
	2	48		55		15		60		23		
	3	11		23		13		12		10		
Blood cultures		48	24	99	26	42	17	68	22	17	12	274
Positive BC		15	11	42	22	18	11	34	16	7	7	116
CRI		6	5	20	17	7	6	14	13	5	5	52
CVAD out		3		10		5		7		3		28
PICU admission		0		1		1		1		1		4
CRI pathogens												
Staphylococcus sp.		1		7		1		5		1		15
Streptococcal sp.		2		5		1		3		2		13
Gram-negative sp.		2		2		1		5		2		12
Mixed Gram pos + neg sp.		0		1		1		1		0		3
Less/non-pathogenic sp.		1		5		3		0		0		9

The maximum body temperature per day was used to assess fever. Days with one recorded body temperature of ≥38.0°C were counted as fever instances. Grades 1, 2, 3 indicate the percentual distribution between Fever grades 1, 2, and 3, respectively. BC, blood culture; CRI, catheter-related infections; CVL, central venous access device; PICU, pediatric intensive care unit; pos, positive; neg, negative; sp., species.

Pain was most severe during the first course and significantly improved during subsequent courses. In our results, 88% of patients experienced grade ≥3 pain in course 1, whereas 41% experienced pain in course 5. Comparable reductions in the proportion of patients experiencing grade ≥3 pain between courses 1 and 5 were seen in the studies of Yu et al. (37%–14%) and Ozkaynak et al. (41%–24%) (2, 9). Decreasing pain scores and intravenous morphine usage in time within and between immunotherapy courses were also observed by Mueller et al. in a study evaluating long-term dinutuximab infusion (LTI, continuous 10-day antibody infusion) (18). Accelerated antibody clearance after repeated administration of dinutuximab may explain the observed decreasing proportion of patients experiencing grade ≥3 pain over the courses (27). However, this explanation of accelerated antibody clearance was contested by another, more recent study (26). The improved pain

TABLE 4 | Immunotherapy-related toxicities leading to dose modifications of dinutuximab.

Course (n=)	100% of cumulative intended dose of dinutuximab administered			≥ 50 to <100% of cumulative intended dose of dinutuximab administered			<50% of cumulative intended dose of dinutuximab administered		
	No. patients	No. patients with dose modifications	Indication for dose modification	No. patients	No. patients with dose modifications	Indication for dose modification	No. patients	No. patients with dose modifications	Indication for dose modification
Course 1 (26)	26 (100%)	8/26	1× AR 3× Cough 2× Pain 1× Pain + Cough 1× Pain + Fever	0 (0%)			0 (0%)		
Course 2 (26)	21 (81%)	3/21	3× Cough	3 (12%)	3/3	1× AR + Fever 1× Cough 1× Hypertension	2 (8%)	2/2	2× CRI
Course 3 (24)	24 (100%)	6/24	6× Cough	0 (0%)			0 (0%)		
Course 4 (24)	19 (79%)	7/19	5× Cough 1× Pain + Cough 1× Hypertension	5 (21%)	5/5	1× AR 2× CRI 1× Fever 1× Hypoxia	0 (0%)		
Course 5 (24)	22 (92%)	4/21	1× AR 1× Cough 1× Fever 1× Pain	1 (4%)	1/1	1× CRI	1 (4%)	1/1	1× Cough

AR, allergic reaction; CRI, catheter-related infection.

tolerance in subsequent cycles could also be explained by the individualization of pain management. The individual and initial pain response would guide the subsequent pain management, making it more effective in subsequent courses.

Eighty-one percent of patients (21/26) suffered from catheter-related infections during anti-GD2 immunotherapy. This percentage is remarkably higher than reported previously (2). Most catheter-related infections (65%) were recorded in the immunotherapy courses containing IL-2 (courses 2 and 4). Previous studies have shown an increase in bacteremia and catheter-related infections in cancer patients receiving IL-2 (28–32). Staphylococcus aureus was cultured in 27% (14/52) of catheter-related infections in our study. This prevalence is strikingly higher than the 5.2% of S. aureus cultured during central line-associated bloodstream infection (CLABSI) episodes in a report on CVAD-related complications in pediatric oncology patients from colleagues at our institution (33). In unpublished data by Van den Bosch et al. on CVAD-related complications in neuroblastoma patients, significantly more S. aureus-CLABSIs and CLABSIs overall were observed in neuroblastoma patients receiving anti-GD2 immunotherapy. Strategies to prevent catheter-related infections have been studied, including the prophylactic use of antibiotics (34, 35) and the use of antibiotic-coated catheters (36). To our knowledge, no studies have examined the benefit of these strategies in this patient population.

The courses containing dinutuximab with IL-2 (courses 2 and 4) were associated with more toxicity than the courses with GM-CSF (courses 1, 3, 5), a result also encountered in other studies (2, 9). Moreover, 19% and 21% of patients did not receive the intended dose of dinutuximab due to toxicity in courses 2 and 4, respectively. In contrast, in the other three courses only in course 5 did 8% of patients not receive the complete dinutuximab dose. In one study, IL-2 was thought to be the causative agent in the majority of fever instances without documented infection (6). In another study by Ladenstein et al, patients were randomly assigned to receive either dinutuximab beta plus IL-2 or dinutuximab beta alone (8). No evidence was found that addition of IL-2 improved outcome. Furthermore, dinutuximab beta plus IL-2 was associated with greater toxicity, more dose modifications and less treatment completion than dinutuximab beta alone, leading the authors to conclude that dinutuximab immunotherapy without IL-2 should be considered standard of care.

The major limitation of our study is the small size of the cohort, making detection of rare complications of treatment less probable. Furthermore, early patients were treated with dinutuximab, while after EMA approval in May 2017 patients were treated with dinutuximab beta. We, however, found no difference in number of grade ≥3 toxicities per patient between the two antibodies and evidence exists that both antibodies have comparable toxicity profiles (37). Lastly, the retrospective nature of our study is a potential source of bias. Although, immunotherapy was newly introduced in our center and all healthcare providers involved were instructed in accurate toxicity recordkeeping, information and selection bias cannot be ruled out completely.

The strength of our study is that all toxicities were uniformly collected, categorized, and graded by a small group with extensive experience in toxicity reporting of cancer treatment in children. We believe that this design in combination with retrospective collection of toxicities from patients' medical files led to more sensitive toxicity collection, and therefore to higher prevalences of toxicities than previously reported (2, 8, 9, 16–18).

We conclude in this single center experience of immunotherapy with dinutuximab, cytokines IL-2 and GM-CSF, and isotretinoin after induction and consolidation according to the DCOG NBL 2009 treatment protocol, that immunotherapy-related toxicity is substantial, but manageable. Future studies are warranted to

optimize the scheduling, anti-GD2 antibody (38), and additive cytokines of immunotherapy with anti-GD2 monoclonal antibodies in high-risk neuroblastoma.

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 the Medical Research Ethics Committee Utrecht (info@metcutrecht.nl).

AUTHOR CONTRIBUTIONS

TB, RL, LA, MM, TW, MD, NE, KK, MN, MG, and GT contributed to the conception and design of the study. TB, RL, and GT organized the database. TB and RL collected the data. TB performed the statistical analysis. TB, RL, and GT wrote the first draft of the manuscript. LA, MM, and TW wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.601076/full#supplementary-material

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Influence of Methylenetetrahydrofolate Reductase C677T and A1298C Polymorphism on High-Dose Methotrexate-Related Toxicities in Pediatric Non-Hodgkin Lymphoma Patients

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Purpose: This retrospective study aimed to investigate the relationships between the methylenetetrahydrofolate reductase (MTHFR) C677T/A1298C and high-dose methotrexate (HD-MTX)-related toxicities in pediatric non-Hodgkin lymphoma (NHL) patients.

Patients and Methods: We reviewed the medical records of 93 NHL patients aged under 18 years who received HD-MTX therapy at the dose of 5 g/m^2 with 24-h infusion at Sun Yat-sen University Cancer Center between 2014 and 2019.

Results: There were 61 males and 32 females, with a median age of 8.8 years (0.9–15.8 years). The tumor types included lymphoblastic lymphoma (n = 38), Burkitt's lymphoma (n = 31), anaplastic large cell lymphoma (n = 18), diffuse large B-cell lymphoma (n = 6). Overall, 355 courses of HD-MTX therapy were prescribed. All patients were rescued with calcium folinate 12 h after the end of MTX infusion. We found that plasma MTX levels > 0.2 μmol/L at 48 h post-infusion increased the risk of developing oral mucositis (2.4% VS. 9.5%, P = 0.018). Also, patients carrying the C677T and T677T genotypes had tendencies to be more susceptible to oral mucositis (P = 0.034). Patients harboring mutant 677T allele were more likely to develop leucopenia (38.5 vs. 50.3%, P = 0.025) and thrombocytopenia (22.0 vs. 32.4%, P = 0.028). For polymorphism A1298C, the mutant genotype played a protective role in vomiting (11.1 vs. 4.3%, P = 0.018) but increased the risk of anemia (23.8 vs. 41.7%, P < 0.001) and leucopenia (38.1 vs. 50.3%, P = 0.021).

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Conclusion: Childhood NHL patients harboring C677T genotype were more vulnerable to oral mucositis, leucopenia, and thrombocytopenia, while those with A1298C genotype were at a decreased risk of vomiting and more likely to develop anemia and leucopenia.

Keywords: MTHFR C677T, MTHFR A1298C, high-dose methotrexate, toxicity, pediatric patients, non-Hodgkin lymphoma

INTRODUCTION

Non-Hodgkin lymphoma (NHL), the fourth most common malignancy across the pediatric age spectrum, is a heterogeneous group of lymphoid malignancies (1, 2). In children, NHL comprises of four main categories, namely, lymphoblastic lymphoma (LBL), Burkitt lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), and anaplastic large cell lymphoma (ALCL) (3). The current overall survival rate of pediatric NHL exceeds 80% due to dramatic progress in developing risk-adapted curative therapy (1), in which methotrexate (MTX) plays a crucial part.

MTX is a well-known folate analog that enters the cell *via* active transport mediated by the reduced folate carrier (RFC1). Subsequently, MTX arrests the folic acid cycle by inhibiting dihydrofolate reductase and influences other important enzymes involved in the folate pathway, such as methylenetetrahydrofolate reductase (MTHFR), an enzyme that interferes with nucleic acid synthesis and favors cell death (4, 5). The imbalance of folate homeostasis may cause DNA synthesis arrest and could disrupt DNA and protein methylation reaction (6). Thus, MTX is used to treat a variety of cancers (4–9). A high-dose MTX (HD-MTX) regimen, referred to the administration of a dosage ranging from 0.5 g/m^2 to 12.0 g/m^2 or even higher, is commonly used to treat childhood acute lymphoblastic leukemia (ALL), lymphoma and pediatric osteosarcoma (5, 10, 11). Despite its wide range of therapeutic efficacy, toxicities related to HD-MTX including reversible myelosuppression, nausea, vomiting, diarrhea, hepatotoxicity, nephrotoxicity, neurotoxicity, and particularly oral mucositis should not be neglected (4, 10–12). HD-MTX-related toxicities can not only lead to interruption or discontinuation of chemotherapy and increase relapse risk, but also affect the quality of life of patients. Therefore, identifying predictors of MTX toxicity is a key to determine effective individual dosage adjustment and to minimize adverse events (13).

The responses to MTX exhibit remarkable interindividual variability, making it difficult to predict who will develop more serious adverse events caused by HD-MTX (10, 12). In addition, accumulating pharmacogenetic studies have revealed that polymorphisms of enzymes involved in the folate pathway could lead to variability in response to MTX- and HD-MTX-related toxicities in various malignancies (12). For example, single nucleotide polymorphism (SNP) in the gene MTHFR, involved in MTX metabolism, demonstrated associations with a variety of nonhematologic and hematologic malignancies (10, 14, 15). MTHFR is a key enzyme for intracellular folate homeostasis and metabolism because it catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF), essential for purine and thymidine synthesis, to 5-methyltetrahydrofolate (5-MTHF), essential for protein synthesis and nucleic acid methylation (4). The two most extensively studied SNPs of MTHFR in relation to the toxicities of MTX are the C677T variant (Ala222Val, rs1801133) and A1298C variant (Glu 429Ala, rs1801131), both dampening enzyme activity by 40–70% (4, 16) (shown in **Figure 1**).

Jared et al. reviewed the studies performed on MTX-induced toxicities across Asian and Caucasian pediatric and adult cancer patients for the MTHFR C677T and A1298C polymorphisms and observed controversial conclusions depending on the patient groups and subgroups investigated in the different systematic reviews as well as the genetic models utilized (7). Also, as most existing studies focused on ALL rather than NHL, there is limited evidence available on the role of C677T and A1298C polymorphisms in HD-MTX-related toxicities in pediatric NHL, with results varying considerably in different studies (10, 14, 16, 17). A multicenter trial NHL-BFM95 with 484 pediatric patients who received 4- or 24-h HD-MTX infusion regimens found that although LBL was significantly associated with MTHFR C677T genotype, this polymorphism did not appear to play a role in the therapy-associated toxicity (18). Noriko Shimasaki also concluded that no significant differences in the development of HD-MTX induced toxicity were observed for the different MTHFR C677T in children with NHL or ALL (19). In contrast, many published studies have suggested significant correlations between the C677T polymorphism and the risk of developing adverse events following MTX-therapy in patients with NHL, including hematologic and non-hematologic toxicity, especially mucositis (7). Very few published information is currently available on the influence of the MTHFR A1298C polymorphism on the development of HD-MTX-associated toxicities and are accompanied with controversial results. Therefore, the primary aim of this retrospective study was to evaluate the influence of C677T and A1298C polymorphisms on HD-MTX-related toxicities in children with NHL treated according to the modified NHL-BFM 95 protocol.

PATIENTS AND METHODS

Patients and Treatment

We reviewed medical records of patients aged ≤ 18 years and diagnosed as NHL at the Sun Yat-sen University Cancer Center (SYSUCC) between 2014 and 2019. The patients were staged according to the new International Pediatric NHL Staging System (20). Only intermediate- and high-risk patients were included. This study was approved by the Institutional Review Board and the Research Ethics Committee of SYSUCC. The ethical approval batch number is B2019-231-01. Besides, this

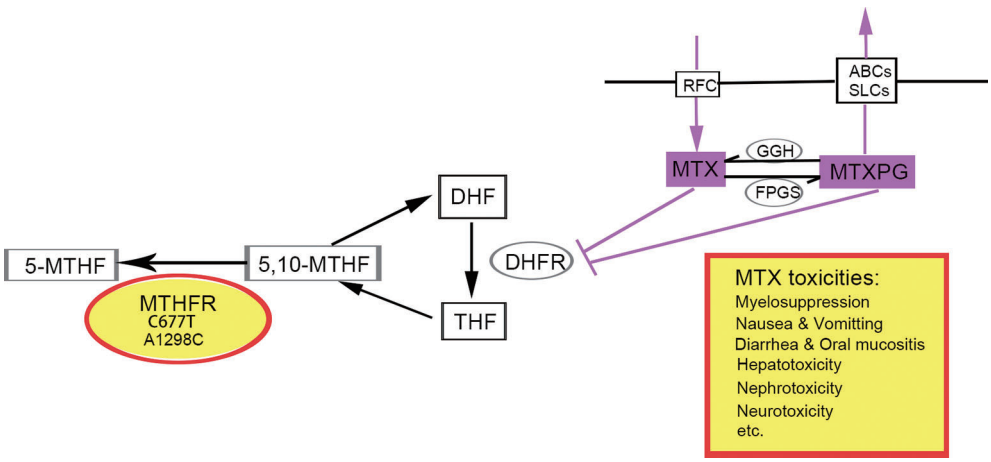

FIGURE 1 | The role of MTHFR polymorphism in folate cycle and MTX metabolism. Polymorphisms in gene MTHFR could reduce the enzyme activity, leading to increased availability of 5,10-MTHF and decreased 5-MTHF. Consequently, the impaired conversion of 5, 10-MTHF to 5-MTHF could modify folate pools and in turn, alter the response of malignant and nonmalignant cells to MTX and potentially aggravates its toxicity. 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; DHF, dihydrofolate; THF, tetrahydrofolate; MTX, methotrexate; MTXPG, methotrexate polyglutamated forms. Transporters, RFC, reduced folate carrier; ABCs, ABC family transporters; SLCs, SLC family transporters. Enzymes, MTHFR, methylenetetrahydrofolate reductase; DHFR, dihydrofolate reductase; GGH, c-glutamyl hydrolase; FPGS, folylpolyglutamyl synthase.

study was registered in the ClinicalTrials.gov and obtained the Clinical Trials. gov ID (NCT042839).

All enrolled patients received treatment according to the modified NHL-BFM95 protocol including MTX therapy at a dose of 5 g/m^2. Each dose of HD-MTX therapy was followed by 6–7 times of calcium folinate (CF) rescue 12 h after the end of the MTX infusion, at a dose of 15 mg/m^2 every 6 h. To maintain the urine pH at approximately 7–8, intravenous hydration and alkalization at the dose of 1,500 ml/m^2 were achieved 12 h prior to the initiation of the HD-MTX administration (D0) and 3,000 ml/m^2 per day lasted for the following 3 days (D1–D3). CF was also given from D1–D3 for mouth rinsing to prevent oral mucositis. We closely monitored the volume and pH of the patients' urine from D0 to D4.

HD-MTX-Related Toxicities

HD-MTX-related toxicities including hematological suppression, hepatotoxicity, nephrotoxicity, oral mucositis, vomiting, and diarrhea were detailly recorded after the MTX treatment until the next course of chemotherapy. Adverse events were graded according to the Common Terminology Criteria for Adverse Events version (CTCAE) 3.0. For analyses, toxicity grades were dichotomized as grade 0, and grade I or II versus III or IV.

MTX Delayed Elimination

Various cutoff points were used to define the presence of clinically meaningfully delayed elimination in different studies (10, 13, 19, 21–23). According to our protocol, the plasma MTX levels were monitored at 0, 24, 48, and 72 h from the initiation of HD-MTX infusion. If the MTX concentration at 48 h was higher than 1 μmol/L, additional CF was performed. Aumente et al. proposed 0.2 μmol/L as a clinical cut-off value for MTX-related toxicities (24). In consensus with other reports (21), we recruited 0.2 μmol/L to define low or high MTX levels at 48 h post-treatment.

Genotypic Polymorphism

The genetic variations of MTHFR were detected by PCR following Sanger sequencing. The PCR reaction was performed as follows: 94°C for 5 min; then 94°C 30 s, 58°C 30 s, 72°C 30 s for 32 cycles and finally, an extension at 72°C for 10 min. The lengths for MTHFR c.677C > T and c.1298 A > C were 246 bp and 256 bp, respectively. The primers were as following: for c.677C > T, Forward: 5′-TGCCCAGTCCCTGTGGTCTC-3′, Reverse: 5′-GGCAAGTGATGCCCATGTCG-3′; and for c.1298 A>C, Forward: 5′ TTTGGGGAGCTGAAGGACTA-3′, Reverse: 5′-ACAGGATGGGGAAGTCACAG-3′. The different genotypes are presented in **Figure 2**.

Statistical Analyses

Statistical analyses were performed with IBM SPSS Statistics 25.0 software (SPSS Inc. Headquarters, 233 S. Wacker Drive, 11th floor, Chicago, Illinois 60606). Chi-square test was used to test deviations from the Hardy-Weinberg (H-W) equilibrium of MTHFR C677T/A1298C genotypes and analyze the associations between MTHFR polymorphism and toxicities. P values < 0.05 were considered significant. All data in our study have been put on record in Research Data Deposit (RDD) at SYSUCC for future reference and obtained the RDD number (RDDA2020001254, https://www.researchdata.org.cn).

RESULTS

Clinical Characteristics of Patients

Table 1 represents the clinical characteristics of the studied group. A total of 93 pediatric patients with the four NHL subtypes were included in this study. The median age of the study cohort was 8.8 years (range: 0.9–15.8 years). There were 61 (65.6%) males and 32

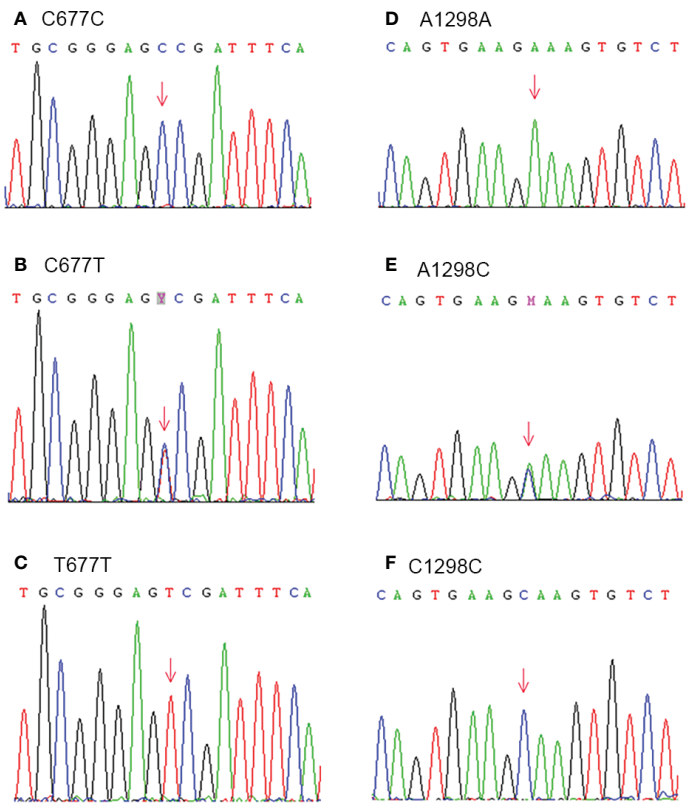

FIGURE 2 | Genetic variations of MTHFR C677T (Ala 222 Val) **(A–C)** and A1298C (Glu 429 Ala) **(D–F)** were detected by PCR following Sanger sequencing.

(34.4%) females. The majority of the patients were diagnosed as LBL (n = 38; 40.9%) and BL (n = 31; 33.3%), and the rest as ALCL (n = 18, 19.4%) and DLBCL (n = 6, 6.5%). Around two-thirds of the patients were diagnosed as stage IV (n = 60; 64.5.6%) and one third as stage III (n = 33; 35.5%) at the time of presentation. They were stratified into an intermediate- (n = 52; 55.9%) or high-risk (n = 41; 44.1%) group. Regarding the MTHFR polymorphism, both C677T and A1298T alleles exhibited the H-W equilibrium in the studied population (677C > T variant: $\chi^2 = 1.2224$, $P = 0.2689$; 1298A > C variant: $\chi^2 = 0.0783$, $P = 0.7796$). Half of the patients (47/93) carried the C677T wild-type genotype (CC), while 35/93 (37.63%) and 11/93 (11.83%) carried the heterozygous (CT) and homozygous (TT) variant genotype, respectively. As for the A1298C polymorphism, one patient failed to be genotyped. Wild-type (AA), heterozygous (CT) and homozygous (TT) variant genotype were observed in 49/93 (53.3%), 37/93 (40.2%) and 6/93 (6.5%) of the subjects, respectively. A total of 355 courses of HD-MTX therapy were analyzed in the present study. The plasma MTX level > 0.2 μmol/L at 48 h post-MTX infusion was observed in 63 (17.7%) courses.

The Role of Plasma MTX Levels in HD-MTX-Related Toxicity

The incidence rate of HD-MTX-related toxicities is shown in **Table 2**. The most frequently observed toxicity was myelosuppression. The results showed that grade III–IV leucopenia and neutropenia occurred in nearly half of the patients. However, the incidence of severe diarrhea (n = 0), nephrotoxicity (n = 0) and hepatotoxicity (n = 6) were too low to be further analyzed. A statistically significant difference was found comparing the incidence rate of oral mucositis in courses with low and high MTX level at 48 h (2.4 vs. 9.5%, $P = 0.018$, **Table 3**).

Associations Between Genetic Polymorphisms and Plasma MTX Levels

We also conducted Chi-square tests to assess the influence of genetic polymorphisms on plasma MTX levels. Plasma MTX levels at 48 h were found to be independent for both C677T and A1298C polymorphisms (**Table 4**). However, MTX levels at 72 h could not be analyzed due to a low occurrence rate of > 0.2 μmol/L.

Associations Between Genetic Polymorphisms and HD-MTX-Related Toxicities

The Chi-square test was used to analyze the associations between MTHFR C677T/A1298C polymorphism and the incidence rate of grade III-IV toxicities (**Table 5**). Polymorphism C677T was found to be significantly correlated with oral mucositis ($P = 0.034$), leucopenia ($P = 0.025$) and thrombocytopenia ($P = 0.028$).

TABLE 1 | Clinical characteristics of the investigated pediatric patients with NHL (n = 93).

Characteristics	No. of patients, n (%)	No. of courses, n (%)
Gender		
Male	61 (65.6)	
Female	32 (34.4)	
NHL subtypes		
LBL	38 (40.9)	
BL	31 (33.3)	
ALCL	18 (19.4)	
DLBCL	6 (6.5)	
Stage		
III	33 (35.5)	
IV	60 (64.5)	
Risk stratification		
Intermediate	52 (55.9)	
High	41 (44.1)	
MTHFR C677T (93/355)		
CC	47 (50.5)	182 (51.3)
CT	35 (37.6)	137 (38.6)
TT	11 (11.8)	36 (10.1)
CT/TT	46 (49.5)	173 (48.7)
MTHFR A1298C (92/352)		
AA	49 (53.3)	189 (53.7)
AC	37 (40.2)	140 (38.9)
CC	6 (6.5)	23 (6.5)
AC/CC	43 (46.7)	163 (45.4)
MTX plasma level at 48 h		
≤ 0.2umol/L		292 (82.3)
> 0.2umol/L		63(17.7)

NHL, Non-Hodgkin Lymphoma; MTHFR, methylenetetrahydrofolate reductase; MTX, methotrexate; h, hour.

TABLE 2 | Prevalence of HD-MTX-related toxicities in children with NHL.

Toxicity	Number of courses (%)	
	Grade I–II	Grade III–IV
Oral mucositis	66(18.6)	13(3.7)
Diarrhea	27(7.6)	0(0)
Vomiting	127(35.7)	30(8.4)
Nephrotoxicity	1(0.3)	0(0)
Hepatotoxicity	53(14.9)	6(1.7)
Anemia	182(51.3)	115(32.3)
Leucopenia	124(34.9)	157(44.3)
Neutropenia	83(23.3)	164(46.2)
Thrombocytopenia	38(10.7)	96(27.0)

HD-MTX, high-dose methotrexate.

Polymorphism A1298C showed associations with vomiting ($P = 0.018$), anemia ($P < 0.001$), and leucopenia ($P = 0.021$).

DISCUSSION

Comparisons of the Clinical Characteristics in Our Study to Previous Reports

Conflicting results are reported in relation to the role of polymorphisms C677T/A1298C in MTX adverse events in NHL (16, 17). The reported conflicting conclusions might result from substantial heterogeneity of the studied population as the MTX toxicity profile could be altered by ethnicity, MTX doses, co-administration of other anticancer agents, renal function, hydration and alkalization, and folinate rescue dosage regimens (10, 11). Unlike most studies including heterogeneous groups of patients, regardless of the type of hematologic malignancy and MTX-based chemotherapy protocols, our study minimized the chances of reporting and detection biases due to random sampling by enrolling a homogenous group of children with NHL treated according to the modified NHL-BFM 95 protocol in China over the past 5 years. Moreover, only patients who received MTX at the dose of 5 g/m^2/24h were enrolled. Thus, the impact on toxicities generated from different doses could be avoided. The gender ratio was approximately 2:1, with more males than females. LBL and BL were observed more frequently than ALCL and DLBCL in Chinese children. Many retrospective analyses in other countries have reported similar clinical characteristics of studied population in pediatric NHL (25–28). Both C677T and A1298T alleles were in the H-W equilibrium with a similar mutant rate (49.5% for C677T and 46.7% for A1298C). Moreover, both genotype frequencies were consistent with those in previous reports (16, 29).

The Role of Plasma MTX Levels in HD-MTX-Related Toxicity

Many studies have suggested that high plasma MTX concentration and prolonged exposure to high levels of MTX were linked to the development of toxicities (12). Accordingly, monitoring plasma MTX during HD-MTX therapy has become mandatory to individually adjust hydration, alkalization, and leucovorin rescue (11, 12). We found that patients with plasma MTX levels > 0.2 μmol/L at 48 h were more pronounced to develop oral mucositis (2.4 vs. 9.5%, $P = 0.018$, **Table 3**). The correlation between higher MTX levels and oral mucositis were also reported in other researches enrolling pediatric leukemia or lymphoma (19, 29) and osteosarcoma (5, 12, 23). One proposed mechanisms for MTX-related mucositis is that MTX may be secreted in the saliva, resulting in elevated direct mucosal toxicity, altered glutathione metabolism, and gastrointestinal microflora, and varied inflammatory responses by proinflammatory cytokines, together with folate metabolic pathway genes, contributing to mucositis (12). Therefore, possible hypothesis would be that patients with higher plasma MTX levels were expected to have higher MTX concentrations secreted into saliva, leading to increased risk of oral mucositis. Based on this assumption, measurements of the MTX plasma level remain vital for monitoring severe mucositis, which would be helpful in personalized management of oral mucositis. Herein, additional preventive strategies like reinforcement of mouth rinsing with CF might be necessary for patients with MTX level > 0.2 μmol/L at 48 h. However, in a study by Yun Jung Choi et al. who analyzed a total of 402 chemotherapy courses in 111 patients with primary central nervous system lymphoma (PCNSL), the authors concluded that MTX-induced oral mucositis occurred independently of the serum MTX level and

TABLE 3 | The relationships between MTX level at 48 h and HD-MTX-related toxicities.

Toxicity (GIII–IV) (No of courses)	MTX level at 48 h		χ^2	*P*
	≤0.2 μmol/L (292)	>0.2 μmol/L (63)		
Oral mucositis (13)	7(2.4%)	6(9.5%)	5.577	0.018
Vomiting (30)	26(8.9%)	4(6.3%)	0.437	0.508
Anemia (115)	96(32.9%)	19(30.2%)	0.175	0.676
Leucopenia (157)	129(44.2%)	28(44.4%)	0.001	0.969
Neutropenia (164)	135(46.2%)	29(46.0%)	0.001	0.977
Thrombocytopenia (96)	80(27.4%)	16(25.4%)	0.105	0.746

that serum MTX concentration might not be a valid marker for predicting mucositis (10). A potential explanation for such inconsistence was the dose of MTX in the protocol for PCNSL was 3.5 g/m^2, much lower than our protocol and the usage of CF for rescue was different.

Associations Between Genetic Polymorphisms and Plasma MTX Levels

Studies on the correlation between polymorphism C677T and MTX levels were more extensive than A1298C. Regarding C677T, Nina Erculj showed that the mean MTX level was significantly higher in pediatric NHL patients of at least one MTHFR 677T allele (6). Previous reports had similar results in lymphoma and ALL (10, 22, 29). On the contrary, other studies indicated that no significant differences in the plasma MTX concentrations were found for the different MTHFR C677T genotype in lymphoma (19), which was in line with our results in regard to which we found that the plasma MTX levels at 48 h were independent in both C677T and A1298C polymorphisms (**Table 4**). However, the intensive rescue therapy in our protocol might have covered the influence of genetic polymorphisms on the plasma MTX levels at 48 h. Moreover, previous pharmacogenomic studies have shown that *MTHFR* was not the only one SNP that could influence the distribution, efficacy, and toxicities of MTX. Other SNPs including *FPGS*, *GGH*, *SLCO1B1*, and *ABCB1* also had similar effects on MTX-pharmacokinetics variability, which might partly account for the aforementioned inconsistence (30).

Associations Between Genetic Polymorphisms and HD-MTX-Related Toxicities

MTHFR C677T

Patients carrying T alleles at MTHFR C677T were reported to experience interruptions in MTX treatment more frequently in childhood ALL or lymphoma (29, 31). In this analysis, we found that although MTHFR C677T/A1298C polymorphism did not significantly affect plasma MTX levels at 48 h, C677T was significantly correlated with oral mucositis (2.2 vs. 3.6 vs. 11.1%, P = 0.034, **Table 5**), leucopenia (38.5 vs. 50.3%, P=0.025, **Table 5**), and thrombocytopenia (22.0 vs. 32.4%, P = 0.028, **Table 5**) and that patients with C677T and T677T genotypes seemed to be more susceptible to those toxicities. The negative effects of polymorphism C677T might be explained by the decreased MTHFR activity. In the case of C677T, C nucleotide is substituted by T nucleotide at position 677, resulting in an amino acid exchange from alanine to valine in the respective amino acid sequence, which lowers the affinity of the enzyme for its cofactor, flavin adenine dinucleotide (5, 14). As a result, individuals in mutant status (C677T and T677T) exhibit 60 and 30% of the normal MTHFR activity, respectively (4, 5). The lowered MTHFR activity increases the availability of 5,10-MTHF but decreases that of 5-MTHF. Consequently, the impaired conversion of 5, 10-MTHF to 5-MTHF could modify folate pools and in turn, alter the response of malignant and nonmalignant cells to MTX and potentially aggravates its toxicity (4, 5, 16). Faganel et al. confirmed that MTX clearance decreased to 73.8% in in childhood ALL and malignant lymphoma with the

TABLE 4 | Associations between genetic polymorphisms and plasma MTX level at 48 h.

Genetic polymorphism (No of courses)	MTX level at 48 h		χ^2	*P*
	≤0.2 μmol/L	>0.2 μmol/L		
MTHFR C677T (355)	292	63		
CC (182)	148(50.7%)	34(50.4%)	0.224	0.894
CT (137)	114(39.0%)	23(36.5%)		
TT (36)	30(10.3%)	6(9.5%)		
CT/TT (173)	144(49.3%)	29(46.0%)	0.224	0.636
MTHFR A1298C (352)	290	62		
AA (189)	157(54.1%)	32(51.6%)	1.275	0.529
AC (140)	114(39.3%)	26(41.9%)		
CC (23)	19(6.6%)	4(6.5%)		
AC/CC (163)	133(45.9%)	30(48.4%)	0.131	0.719

TABLE 5 | Associations between genetic polymorphisms and HD-MTX-related toxicities.

Toxicity (GIII–IV)	MTHFR C677T			χ^2	*P*	MTHFR A1298C			χ^2	*P*
(No. of courses)	CC(182)	CT(137)	TT(36)			AA(189)	AC(140)	CC(23)		
	CC(182)	CT/TT(173)				AA(189)	AC/CC(163)			
Oral mucositis (13)	4(2.2%)	5(3.6%)	4(11.1%)	6.768	0.034	4(2.1%)	8(5.7%)	1(4.3%)	2.957	0.228
	4(2.2%)	9(5.2%)		2.27	0.132	4(2.1%)	9(5.5%)		2.85	0.091
Vomiting (30)	14(7.7%)	11(8.0%)	5(13.9%)	1.543	0.462	21(11.1%)	7(5.0%)	0(0%)	6.229	0.044
	14(7.7%)	16(9.2%)		0.28	0.598	21(11.1%)	7(4.3%)		5.55	0.018
Anemia (115)	56(30.8%)	45(32.9%)	14(38.9%)	0.926	0.630	45(23.8%)	58(41.4%)	10(43.5%)	12.915	0.002
	56(30.8%)	59(34.1%)		0.45	0.502	45(23.8%)	68(41.7%)		12.9	0.0003
Leucopenia (157)	70(38.5%)	64(46.7%)	23(63.9%)	8.439	0.015	72(38.1%)	71(50.7%)	11(47.8%)	5.370	0.068
	70(38.5%)	87(50.3%)		5.03	0.025	72(38.1%)	82(50.3%)		5.3	0.021
Neutropenia (164)	78(42.9%)	65(47.4%)	21(58.3%)	3.036	0.219	83(43.9%)	69(49.3%)	10(43.5%)	0.998	0.607
	78(42.9%)	86(49.7)		1.68	0.195	83(43.9%)	79(48.5%)		0.73	0.393
Thrombocytopenia (96)	40(22.0%)	39(28.5%)	17(47.2%)	9.938	0.007	46(24.3%)	44(31.4%)	3(13.0%)	4.345	0.114
	40(22.0%)	56(32.4%)		4.86	0.028	46(24.3%)	47(28.8%)		0.91	0.34

MTHFR 677TT genotype (29). Therefore, patients carrying variant alleles might have an increased risk of developing higher intolerance to MTX (14).

The elimination of MTX mainly relies on kidney and liver. However, MTX could be accumulated in the third space or binds to protein, from which MTX clearance would be much slower (32). Therefore, in the case of oral mucositis, we hypothesized although the T allele at MTHFR C677T had no impact on the plasma MTX levels, it would ultimately lead to the delayed MTX elimination in the saliva, provoking higher risks of oral mucositis. However, Yun Jung Choi et al. demonstrated that the incidence of and oral mucositis requiring treatment was highest among patients with the wild type in CNS lymphoma (10). A possible reason might lie in the different criteria used for severe adverse events and different analytical methods utilized. Therefore, more attention should be paid to patients with T alleles. Similarly, the variant C allele might have impact on the MTX levels in the bone marrow rather than the blood, increasing the risk of anemia and thrombocytopenia.

Our results were in consensus with most existing studies. Mohammad et al. demonstrated that MTHFR 677C > T polymorphism was an independent marker for predicting MTX-associated hematological toxicity in NHL (14). Angelo et al. showed that pediatric NHL patients harboring 677T allele had an approximately six-fold greater risk of developing hematological toxicity compared with wild-type carriers (9). Nina Erculj et al. reported that compared to patients with wild-type genotype, MTHFR 677T allele carrier had higher odds of leucopoenia and thrombocytopenia, probably through modulating MTX pharmacokinetics in pediatric NHL patients (6). Moreover, Barbara et al. implied that the MTHFR 677TT polymorphism was associated with an increased incidence of mucositis after HD-MTX treatment in lymphoma (29). Besides, Donato et al. revealed that adult NHL patients carrying 677TT genotype significantly increased the risk of developing mucositis and thrombocytopenia (17). Based on our clinical observations, for patients with MTHFR 677T allele, we guess reinforcement of mouth rinsing with CF could reduce the risk of developing oral mucositis and the preventive use of recombinant human granulocyte colony-stimulating factor (G-CSF) may decrease the incidence of leucopenia. However, further clinical research is necessary to find out whether CF and G-CSF could improve or reverse the effects of T alleles.

MTHFR A1298C

Regarding the influence of A1298C polymorphism on MTX toxicities, Nina Erculj et al. confirmed that MTHFR 1298A > C did not show any associations with myelosuppression, hepatotoxicity, nephrotoxicity, gastrointestinal toxicity and mucositis in pediatric NHL (6). However, other authors reported significant correlations between MTHFR A1298C and MTX toxicities but have not validated whether it plays a protective role against bone marrow and hepatic toxicity in children with ALL or NHL. Barbara et al. found that lymphoma patients homozygous for the variant MTHFR were at a decreased risk for leucopenia (29). Donato et al. demonstrated that NHL patients with 1298CC genotype were at a higher risk for developing mucositis (17). In a recent study by Goekkurt et al., the authors described that A1298C polymorphism seemed to be an independent predictor for hepatic toxicity with a higher risk for the 1298CC genotype with respect to AC and AA genotypes (33). Interestingly, unlike C677T, polymorphism A1298C was not suggested to increase the risk of oral mucositis but still correlated with hematological suppression in the present study. The mutant genotypes increased the risk of anemia (23.8 vs. 41.7%, $P < 0.001$, **Table 5**) and leucopenia (38.1 vs. 50.3%, $P = 0.021$, **Table 5**). However, the fact should not be neglected that the enzymatic activity of MTHFR is affected by A1298C polymorphism to a less extent than C677T, and that the remaining activity in the C1298C homozygous genotype still represents 60% of the normal status despite of the substitution of glutamate for alanine in the amino acid sequence (4, 14). Furthermore, our data indicated significant correlations between MTHFR A1298C polymorphism and vomiting. The mutant genotypes played a protective role in vomiting (11.1 vs. 4.3%, $P = 0.018$, **Table 5**). Intestinal toxicity is a common adverse event of MTX and may influence the entire intestinal tract, contributing to symptoms like nausea, bloating, and diarrhea.

The gut microbiome has been reported to be able to modulate the host response to chemotherapeutic drugs and be associated with the intestinal toxicity of MTX (34, 35). We speculated that there might be an undiscovered positive role of C alleles at MTHFR A1298C on the MTX-gut microbiome interaction, relieving the symptomatic vomiting. Therefore, preventive administration of anti-vomiting agents would be more necessary for patients with wild-type genotype while G-CSF for patients with mutant genotype.

Limitations

There are several limitations in this study. The major limitation is its retrospective nature. Additionally, MTX metabolism is influenced by not merely MTHFRC677T/A1298C but also many other genetic polymorphisms including SLC19A1, MTHFRG1793A (Arg594Gln) and ABCB1 (29, 36). Therefore, only taking MTHFRC677T/A1298C into consideration might not be convincing enough to explore their relationship with HD-MTX toxicities. Moreover, this is a single center study.

CONCLUSION

Our results may provide a useful resource for clinicians to consider personalized medicine. Our data showed that patients with MTX levels higher than 0.2 μmol/L at 48 h were more vulnerable to oral mucositis. Analyzing MTHFR C677T and A1298C polymorphism prior to treatment might be useful for monitoring MTX-related adverse events in childhood NHL. Patients harboring mutant C677T genotype were more vulnerable to oral mucositis, leucopenia, and thrombocytopenia while those with mutant A1298C genotype were more likely to develop anemia and leucopenia but less susceptible to vomiting. Nevertheless, further prospective multicenter studies are required to validate the predictive significance of these associations.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: www.researchdata.org.cn, RDDA2020001254.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board and the research ethics committee of the Sun Yat-sen University Cancer Center (SYSUCC). 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

SL, XZ, WL, and HC contributed equally to this work in methodology, software, formal analysis, investigation, data curation, writing—original draft, and writing—review and editing. DZ, ZZ, FS, JH, JZ, and JW worked collaboratively in investigation and data curation. YZ and XS contributed equally to this work in conceptualization, supervision, and project administration. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Fertility Preservation in Female Pediatric Patients With Cancer: A Clinical and Regulatory Issue

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Fertility preservation represents one important goal of cancer patients' management due to the high impact on health and quality of life of survivors. The available preventive measures cannot be performed in all patients and are not feasible in all health-care facilities. Therefore, the pharmacological treatment with GnRHa has become a valuable non-invasive and well-tolerated alternative, especially in those who cannot access to cryopreservation options due to clinical and/or logistic issues. Supporting data demonstrate a significant advantage for the survivors who received GnRHa in the long-term maintenance of ovarian function and preservation of fertility. The prevention of the risk of ovarian failure with GnRHa is a typical off-label use, defined as the administration of a medicinal product not in accordance with the authorized product information. Italy has officially recognized the off-label use of GnRHa in adult women at risk of premature and permanent menopause following chemotherapy. However, fertility preservation still represents an unmet medical need in adolescents who cannot access to other treatment options.

Keywords: GnRHa, chemotherapy, adverse event, off-label, regulatory issue

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INTRODUCTION

In Europe, nearly 80% of children and adolescents with cancer treated on current protocols survive at least 5 years on average (1). Improved survival rates have increased the number of childhood cancer survivors (CCSs) entering adulthood after treatment for malignancy, which account for 0.1–0.15% of the general population (2). The high survival rate in children and adolescents is accompanied by a substantial risk of late adverse events (LAEs). Above all, treatment may interfere with physiological growth and development and have an important impact on health status later in life, whilst some late toxicities may cause premature death. Fertility is one of the most important concerns of CCSs (3, 4). The occurrence of fertility impairment in female—reduced pregnancy rates and increased risk of early menopause—after pelvic, abdominal, or spinal radiotherapy, total body irradiation, or chemotherapy regimens containing alkylant agents during childhood and adolescence has been widely documented (5–8).

Treatments may deplete or accelerate the decline of the non-renewable pool of primordial follicles in the ovary leading to POF and infertility (9, 10). Gonadal toxicity is affected by type, doses and length of therapy (11, 12), and by age at treatment (females treated at a younger age are less likely to develop POF, probably because of a higher number of primordial follicles at the time of treatment). POF results in a reduced fertile lifespan and associated risk for involuntary childlessness, which can negatively impact the quality of life (13–15), but also accelerates the risk of developing menopause-associated conditions, such as osteoporosis and cardiovascular disease (16). Therefore, fertility preservation (FP) represents an important issue for oncologists, fertility specialists and patients. Most recent guidelines (12, 17, 18) state FP should be discussed with the parents (or guardians) of adolescents soon after diagnosis and before starting anticancer treatments.

In pubertal and postpubertal adolescents, oocyte cryopreservation is one of the available option for FP during gonadotoxic chemotherapy (18) (**Table 1**). Oocyte cryopreservation (21) requires ovarian stimulation with gonadotropin hormone, ultrasound-assisted oocyte collection, and selection and freezing of oocytes (22). There are many barriers to the adoption of this option into standard of care. First, oocyte cryopreservation should be carried out before starting chemotherapy and requires time (17) for ovarian stimulation and follicular growing, with consequent delay in the initiation of oncological treatment. This can be a problem especially in pediatric cancers, which often require urgency to start treatment. The random-start controlled ovarian stimulation protocol, providing for a stimulation at any time of the menstrual cycle, can reduce the time needed for cryopreservation, but for patients with very aggressive diseases no delay is allowed (23). Moreover, this technique is associated with additional challenges as it requires to acquire oocytes through transvaginal approach (24), a painful procedure to be performed with sedation and with specialized equipment, which is not often available in pediatric

TABLE 1 | Treatment options for fertility preservation.

Technique	Definition	Advantages	Disadvantages	Experimental
Oocyte cryopreservation	Controlled ovarian stimulation, followed by oocyte retrieval and cryoconservation for future use	- well-established fertility preservation technique - no ethical issues	- time required for ovarian stimulation - risk of overstimulation - invasive procedure for oocyte retrieval (day surgery) - not recommended in women with hormone-sensitive cancers - not possible for prepubertal girls - need of a male partner/donor for oocyte fertilization prior to implantation - high cost	No
Embryo cryopreservation	Controlled ovarian stimulation, followed by oocyte retrieval, in vitro fertilization and embryo cryopreservation for future use (19, 20).	- well-established fertility preservation technique - good embryo survival to thawing - direct transfer into the uterus after thawing	- ethical issues regarding embryo disposition - time required for ovarian stimulation and subsequent delay in timely cancer treatment - risk of overstimulation - invasive procedure for oocyte retrieval (day surgery) - not recommended in women with hormone-sensitive cancers - not possible for prepubertal girls - need of a male partner/donor - high cost	No*
Ovarian Tissue cryopreservation	Surgical retrieval of ovarian tissue, cryopreservation of the tissue and subsequent reimplantation once patient is disease-free	- feasible for prepubertal children - does not require hormonal stimulation - can be planned shortly after diagnosis of malignant disease	- surgical procedure under general anesthesia - risk of the re-introduction of carcinogenic cells - risk of malignant transformation of the ovarian tissue - risk of ischemic damage to the tissue - limited availability of centers with adequate cryoconservation competences and able to perform the most sensitive and updated histological analysis techniques before transplantation to avoid relapses - high cost	Yes
Ovarian suppression with GnRHa	Concomitant use of gonadotropin-releasing hormone analogs (triptorelin, goserelin, leuprolide) during the course of chemotherapy to induce a prepubertal hormonal milieu and preserve the ovarian function	- not invasive - does not need delaying in oncologic therapy - can be used in association with cryopreservation techniques - reduce the risk of hypermenorrhea associated with hematologic malignancies or myelosuppressive treatments - low cost	- symptoms of estrogenic deprivation - transient alterations of bone metabolism not significant for therapy duration < 6 months - limited clinical evidences in patients with disease other than breast cancer	Yes**

**Forbidden in Italy (Law 40/2003); **Reimbursed in Italy for oncologic adult patients according to Law 648/96.*

hospitals. Not less important is its emotional and psychological impact for most adolescents, despite their sexual maturity.

Ovarian tissue cryopreservation (OTC), although still considered as experimental, is commonly proposed as an alternative to oocyte cryopreservation (18). Cryopreservation of the ovarian tissue requires a laparoscopic procedure under general anesthesia and the subsequent freezing of the tissue that contains most primordial follicles (25). Differently from oocyte cryopreservation, OTC can be performed at any time with less delay in starting cancer therapy. Once the patient is disease-free, an autotransplantation can be carried out. Major issues of OTC include ischemic damage to the tissue and the theoretical risk of reintroducing malignant cells, especially. Indeed, transplantation is not in patients with diseases associated with a high risk of ovarian metastases (26, 27). Another limitation is represented by the availability of a center with adequate cryoconservation competences and able to perform the most sensitive and updated histological analysis before transplantation to avoid relapses (28).

An alternative, that could represent a more accessible FP option burdened by less discomfort for postpubertal patients, could be the concomitant use of gonadotropin-releasing hormone (GnRH) analogs (triptorelin, goserelin, leuprolide) during the course of chemotherapy (18, 29, 30). This non-invasive and less expensive approach could allow preventing POF in cancer survivors and has been recently integrated in clinical guidelines (17, 18, 31–34). In particular, the updated European Society for Medical Oncology (ESMO) and American Society of Clinical Oncology (ASCO) recommendations state that GnRHa should be used in addition to the other options (17, 35).

It is noteworthy that in adolescents and young women with malignancies GnRHa are widely used as an alternative to estro-progestinic combinations for menstrual suppression (36–41), in order to reduce the risk of heavy menstrual bleeding associated with hematologic malignancies or myelosuppression induced by chemotherapy. In this setting, despite significant side-effects simulating the physiology of menopause and the risk of loss of bone mineral density with prolonged use (usually > 6 months) (42), GnRHa are well tolerated and effective option for menses suppression (43, 44) and are generally preferred to oral contraceptives for several reasons. Oral contraceptives have some disadvantages such as the daily regimen, in contrast to the monthly administration of GnRHa. Furthermore, the efficacy of oral contraceptives can be reduced by an erratic absorption due to mucositis, diarrhea, and emesis. Moreover, the use of estrogen-based oral contraceptives for menstrual suppression is associated with an increased risk of venous thromboembolism (41, 45). However, one of the most important reason for choosing GnRHa is represented by their possible gonadal protective effect (41).

THE CLINICAL PERSPECTIVE: CONCLUDED AND ONGOING TRIALS

The rational for the use of GnRH analogs (GnRHa) for the reduction of ovarian toxicity is based on the observation that chemotherapy mostly affects tissues with rapid cellular turn-over, as gonadal one (46). Moreover, the gonadotoxicity is lower in prepubertal girls than in adult women (47, 48), probably as a consequence of their higher ovarian reserve, in addition to the hypogonadotropic prepubertal milieu. This led to the speculation that ovarian suppression in postpubertal female patients might mitigate the adverse effects of treatment on ovarian function (49). Preclinical data have confirmed the efficacy of GnRHa in reducing cyclophosphamide-induced gonadotoxicity (50–53). A prospective randomized study in primates demonstrated that co-treatment with GnRHa significantly decreased the rate of follicular decline and the total number of primordial follicles lost compared with cyclophosphamide alone (53).

GnRHa may protect the ovaries against chemotherapy-induced damage through the inhibition of the hypothalamic-pituitary-ovarian axis with the induction of a prepubertal state (54). The increased rate of non-resting follicles loss leads to a decrease in the secretion of sex steroids and inhibins produced by these follicles at different stages of maturation and differentiation. The resultant low systemic concentrations of these endogen molecules induce a feedback on the hypothalamus and pituitary gland, increasing the gonadotropins secretion, mainly follicle-stimulating hormone (FSH) (54), which enhance the follicle recruitment and maturation. These growing follicles are more exposed to the gonadotoxic effects, ending in an accelerated rate of follicular apoptosis and degeneration. This vicious cycle may be interrupted by preventing the increase in FSH through the GnRHa administration (55). Moreover, GnRHa may exert beneficial effects through the decrease in utero-ovarian perfusion resulting from the hypoestrogenic state (56, 57), with lower exposure of the ovaries to chemotherapy. In addition, human gonads express GnRH receptors (58–63), which activation may decrease apoptosis (61). Other hypothetical gonadoprotective mechanisms include an increased formation of intragonadal antiapoptotic molecules (64, 65) and protection of the undifferentiated germline stem cells (54), with the latter mechanism not yet tested under an experimental model.

Several randomized clinical trials (RCTs) have shown a clear benefit and a good safety profile of GnRHa in the prevention of chemotherapy damage in pre-menopausal women aged 18–45 years with breast cancer (66–76) (**Supplementary 1**). In these RCTs, patients with normal ovarian function were randomized to receive chemotherapy plus GnRHa or alone. The study population was heterogeneous, with different age, chemotherapy regimens, selection criteria and follow-up duration. The markers for FP were mainly represented by the return of ovarian function, and the assessment of ovarian reserve by measurement of hormone levels. Only few studies evaluated the long-term pregnancy rate in survivors, the most appropriate marker of fertility that requires a prolonged follow-up, especially in a young population. The three larger phase III RCTs [US POEMS–SWOG/S0230 (73, 74), Italian PROMISE–GIM6 (68) and Anglo Celtic Group OPTION trial (75)], demonstrated a statistically significant reduction in POF and a significant increase in pregnancy rate in the GnRHa arms. A large meta-analysis of 12 RCTs including patients with breast cancer (77) confirmed these positive findings showing a significant reduction of the risk of

TABLE 2 | Studies involving adolescent patients.

Author (Study Design)	Number of Patients (Disease)	Age range (years)	Chemotherapy protocol	GnRHa (dosage/posology-duration)	Treatment arms (patients per arm)	Outcome measures	Follow-up duration
Gilani et al. (82) (Phase III, randomized clinical trial)	30 (Ovarian malignancies)	12–40	Three to seven courses of one of the following regimens: VAC; BEP; TC; CP	Triptorelin depot, 3.75 mg, i.m., administered 7 days before starting chemotherapy and every 28 days during chemotherapy treatment	1. Chemotherapy + triptorelin (15) 2. Chemotherapy alone (15)	Resumption of menses and serum FSH level < 20 mIU/ml at 6 months post- chemotherapy	6 months
Cheng et al. (98) (Phase II, open label, non-randomized clinical trial)	60 (Hematologic malignancies, mainly HD and AML)	15–39	HSCT myeloablative (cyclophosphamide +TBI; etoposide+TBI; busulfan+ cyclophosphamide; busulfan+ melphalan; busulfan+ fludarabine; carmustine, etoposide, cytarabine, and melphalan) or non-myeloablative (fludarabine+melphalan; fludarabine+cyclophosphamide; cyclophosphamide; melphalan+arsenic) conditioning regimens	Leuprolide 22.5 mg in a 3-month depot i.m. injection, given within 2 months before stem cell transplantation. The second dose of leuprolide was given 3 months after the first injection.	1. Leuprolide + HSCT conditioning regimen (60)	Resumption of menses and monitoring of FSH, LH, and estradiol levels	355 days (median; range 102–1,676 days)
Castelo-Branco et al. (89) (Open label, comparative, non-randomized, prospective study)	56 (HD)	14–45	C-MOPP-ABV; C-MOPP-ABV+RTP; C-MOPP-ABV+MINE-ESHAP+ASCT; C-MOPP+ABVD; ABVD; ABVD+RTP; ABVD+MINE-ESHAP +ASCT; ABVD+RTP+MINE+ESHAP+ ASCT; C-MOPP/ABV+RTP+ ASCT; ABVD +C-MOPP+RTP	Triptorelin depot, 3.75 mg, i.m., 1–2 weeks before starting chemotherapy and every 4 weeks during chemotherapy treatment + 2.5 mg daily tibolone	1. Chemotherapy + triptorelin + tibolone (30) 2. Chemotherapy alone (26)	Resumption of menses; monitoring of serum levels of FSH, LH, 17β-E2, and inhibin B during and after chemotherapy; bone mineral density loss monitoring	NM
Blumenfeld et al. (99) (Prospective non-randomized study with historical control)	36 (HD and NHD)	15–40	MOPP/ABV(D), CHOP, C-MOPP, or ABV with or without radiotherapy	Triptorelin depot, 3.75 mg i.m., administered 7–10 days before starting chemotherapy and monthly during chemotherapy treatment, until its conclusion or for a maximum of 6 months	1. Chemotherapy + triptorelin (18) 2. Chemotherapy alone (18)	Resumption of menses and regular cyclic ovarian function; ultrasonographic monitoring of ovarian folliculogenesis and ovulation; monitoring of FSH, LH, estradiol, progesterone, and prolactin levels	up to 4 years (up to 8 years for historical control group)
Blumenfeld et al. (92) (Prospective non-randomized study with concurrent and historical controls)	111 (HD)	15–40	ABVD; MOPP/ABV(D); Standard BEACOPP; Escalated BEACOPP	Triptorelin depot, 3.75 mg i.m., administered 2-7 days before starting chemotherapy and monthly during chemotherapy treatment up to a maximum of 6 months	1. Chemotherapy + Triptorelin (65); 2. Chemotherapy alone (46)	Resumption of menses and cyclic ovarian function; incidence of spontaneous pregnancies; primordial follicle count on both ovaries; FSH, LH, E2 and P levels monitoring	8 years (mean–range 2–15 years)
Pereyra Pacheco et al. (88) (Prospective non-randomized study with historical controls)	16* (HD, NHD, AML)	14.7–20	ICE+BMT; CAVPE+BMT; CCOPP+CAVPE+ESHAP+ ICE+ BMT; CAPVE+BMT; CAVPE+ICE +BMT; CVPP; ABVD; CVPP X 1 + ABVD X 5	Leuprolide depot, 3.75 mg i.m., starting 5-7 days before chemotherapy and monthly during chemotherapy treatment, until 30 days after the end of treatment	1. Chemotherapy (±BMT) + leuprolide (12) 2. Chemotherapy (±BMT) alone (4)*	Resumption of menses and regular cyclic ovarian function; FSH, LH, and estrogens level monitoring; incidence of spontaneous pregnancies; bone density loss monitoring	up to 5 years (up to 6 years in control group)
Blumenfeld et al. (94) (Prospective non-randomized study)	83 (HD, NHL, leukemias and other diseases)	14–40	Conditioning chemotherapy (busulfan and cyclophosphamide; TBI and etoposide; busulfan, cyclophosphamide, fludarabine and antithymocyte globulin; BEAC; or BEAM) before stem cell transplantation	Triptorelin depot, 3.75 mg i.m. 7–14 days before starting gonadotoxic therapy and monthly during chemotherapy	1. Conditioning chemotherapy + triptorelin (47) 2. Conditioning chemotherapy alone (36)	Resumption of menses and cyclic ovarian function; FSH, LH, E2, and P levels monitoring; ultrasonographic monitoring of ovarian antral follicles	7 years (median-range 2–13 years) for triptorelin arm and 8 years (median -range 2-13 years) for controls

(Continued)

TABLE 2 | Continued

Author (Study Design)	Number of Patients (Disease)	Age range (years)	Chemotherapy protocol	GnRHa (dosage/posology-duration)	Treatment arms (patients per arm)	Outcome measures	Follow-up duration
						and stimulated endometrium; spontaneous pregnancies	
Blumenfeld et al. (96) (Retrospective case-control study)	474 (HD, NHL, leukemias and other diseases)	14–40	ABVD (± MOPP); BEACOPP/escalated BEACOPP; BMT; CHOP/CVAD	Triptorelin depot, 3.75 mg i.m., administered 7-14 days before starting chemotherapy and monthly during chemotherapy treatment	1. Chemotherapy + triptorelin (286) 2. Chemotherapy alone (188)	Spontaneous pregnancy rate; resumption of menses; FSH, LH, E2, and P levels monitoring; ultrasonographic monitoring of ovarian folliculogenesis and ovulation	up to 25 years (range 2–25 years)
Meli et al. (100) (Retrospective observational study)	36 (ALL; AML; HD; NHL; solid tumors)	11–18	Chemotherapy protocols containing alkylating agents (cyclophosphamide; procarbazine; ifosfamide; carmustine; mitoxantrone; melphalan; busulfan; thiotepa; treosulfan; muphoren) ± radiotherapy or high-dose chemotherapy and HSCT	Triptorelin depot, 3.75 mg i.m. monthly during chemotherapy or 11.25 mg every 3 months, for 3 to 12 months (median, 8 months)	1. Chemotherapy + triptorelin (27)	Resumption of regular spontaneous menstrual cycle; FSH, LH, E2 and P levels monitoring; ultrasonographic visualization of ovarian follicles or corpora lutea; spontaneous pregnancies	7 years from diagnosis (median -range 2-18 years); 5 years from stop therapy (median -range 1–17 years)
Gini et al. (101) (Retrospective and Cross-sectional study)	97 (HD or NHL)	16–50	ABVD or ABVD-like regimens, RCHOP, or VACOP-B ± radiotherapy; second-line regimens followed by HSCT	NM	1. Chemotherapy + oral contraceptives or GnRHa 2. Chemotherapy alone	Resumption of menstrual activity; use of oral contraceptives or GnRHa during chemotherapy; number of pregnancies and offsprings after therapy	NM

**In the study there is another historical control group of premenarchal patient not treated with GnRHa, not mentioned here (n=5).*

NM, not mentioned; HD, Hodgkin's disease; NHL, non-Hodgkin lymphoma; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; ABV, doxorubicin, bleomycin, and vinblastine; ABVD, doxorubicin, bleomycin, vinblastine, dacarbazine; ASCT, autologous stem cell transplantation; BEAC, BCNU, etoposide, cytarabine, cyclophosphamide; BEACOPP, bleomycin, etoposide, adriamycin, cyclophosphamide, vincristine, procarbazine, prednisone; BEAM, BCNU, etoposide, cytarabine, melphalan; BEP, bleomycin, etoposide, cisplatin; BMT, bone marrow transplantation; CAVPE, cyclophosphamide, adriamycin, vincristine, prednisone, etoposide; CCOPP, CCNU (lomustine), cyclophosphamide, vincristine, procarbacine, prednisone; CHOP, cyclophosphamide, adriamycin, vincristine, prednisone; C-MOPP, cyclophosphamide, vincristine, procarbazine, prednisolone; CP, taxol, cisplatin; CVAD, cyclophosphamide, vincristine, doxorubicin, dexamethasone; CVPP, cyclophosphamide, vinblastine, procarbazine, prednisone; ESHAP, etoposide, methylprednisolone, high-dose cytarabine, and cisplatin; HSCT, hematopoietic stem cell transplantation; ICE, ifosfamide, carboplatin, etoposide; MINE, mesna, ifosfamide, mitoxantrone, and etoposide; MOPP, mechloroethamine; vincristine; procarbazine, prednisolone; R-CHOP, rituximab, cyclophosphamide, adriamycin, vincristine, prednisone; RTP, radiotherapy; TBI, total body irradiation; TC, taxol, carboplatin; VAC, vincristine, dactinomycine, cyclophosphamide; VACOP-B, etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin.

POF at 12 months from the end of chemotherapy, and a greater number of pregnancies. The efficacy and safety of GnRHa as a clinical option to reduce POF and improve fertility was further confirmed by a recent meta-analysis and systematic review (78), that showed a statistically greater number of pregnancies in the GnRHa group and no differences in progression-free survival.

The similar or even improved survival outcomes of premenopausal breast cancer patients who received GnRHa, reported by the main RCTs and meta-analysis (79) dispelled the safety concern on the potential antagonism between concurrent GnRHa and chemotherapy.

Moreover, no significant increase in the occurrence of GnRHa-associated toxicities (e.g. hot flashes, sweating, headache, vaginal dryness, and thromboembolic events) has been reported (68, 73).

In the light of these results, the most updated guidelines consider temporary ovarian suppression with GnRHa during chemotherapy as an option to be discussed with breast cancer patients interested in preserving ovarian function (17, 80, 81).

Currently, limited evidence exists on the role of this strategy in women diagnosed with tumors other than breast cancer. One randomized trial has assessed the temporary ovarian suppression with GnRHa in 30 young patients with ovarian cancer (82). The study showed a significant reduction in the risk of chemotherapy-induced POF, although no information on post-treatment pregnancies was reported.

Randomized trials performed in women with hematological malignancies showed no protective effect of GnRHa or suggested a partial protective effect with only a delaying in the appearance of POF (83–86). It is noteworthy that all these studies had a small sample size and were not powered to find a possible advantage of GnRHa. Indeed, when the gonadotoxicity is either very low or very high (>90%) the needed power to detect a difference between the study arms requires hundreds of patients. However, other large retrospective or prospective studies and case series have shown a potential protective effect of GnRHa during chemotherapy also in women with hematological malignancies (87–97).

Nevertheless, at present, only very limited evidences from RCTs regarding the use of GnRHa in adolescents with cancer are available (**Table 2**, **Supplementary 2**). This could be due, at least in part, to the difficulty of carrying out, in such a young population, clinical trials with a follow-up long enough to allow the evaluation of reliable fertility preservation indicators, such as pregnancy rate. Indeed, menses resumption is an indirect marker of fertility, but patients resuming menses may have a subclinical and irreversible depletion of ovarian reserve and may experience early menopause (102, 103). The only prospective phase III RCT including postmenarchal adolescent patients, affected by ovarian malignancy, demonstrated the gonadoprotective effect of GnRHa even in the younger population (82). Six months after chemotherapy, all the patients in the GnRHa group had normal menstrual bleeding and normal titer of FSH/LH, whereas 33% in the control group had amenorrhea and POF. A phase II trial evaluated the gonadoprotective effect of leuprolide in adolescent and young women affected by hematologic malignancies who underwent to hematopoietic stem cell transplantation (HSCT) (98). In this case only seven patients (16%) regained ovarian function and leuprolide failed to significantly preserve fertility.

However, such poor outcome could be explained by the fact that almost all patients received at least one prior chemotherapy regimen (median number before HSCT = 2), and 12 patients also received prior local radiation. Therefore, ovarian reserve was probably affected by previous gonadotoxic exposure. Another limitation was the use of a very high dosage of GnRHa. Whereas previous studies used monthly 3.75 mg triptorelin or monthly 3.6 mg goserelin or 11.25 mg leuprolide every 3 months, in this study 22.5 mg leuprolide were administered in 3-month depot injection within 2 months of HSCT. The high doses used in this trial led to intolerable side effects and treatment discontinuation in some patients (104).

Several prospective non-randomized studies have shown the ability of GnRHa to provide a powerful instrument for protection of the ovarian function even in adolescents with hematological malignancies (88, 96, 99, 101, 105).

In a prospective case series with control (88), postpubertal adolescents with normal ovarian function who received monthly leuprolide before and during polychemotherapy for lymphoma, resumed their menstrual cycles and ovulation. After a follow-up of five years, three normal pregnancies were reported. In contrast, patients in the control group had permanent hypergonadotropic amenorrhea.

A long-term follow-up analysis (up to 15 years) of adolescent and young adult with Hodgkin lymphoma co-treated with triptorelin, confirmed the gonadoprotective effect of GnRHa (92). Indeed 96.9% in the GnRHa group resumed ovulation and regular menses, throughout a median follow-up of 8 years (range 2–15), compared with 63% in the control group.

Interestingly, a case report (91, 106) documented four spontaneous pregnancies and two successful deliveries in a patient previously undergoing repeated SCTs and monthly GnRHa co-treatment. SCT almost invariably induces POF owing to higher chemotherapy doses and possible total-body irradiation (107, 108). The estimated odds for spontaneous conception after two SCTs became negligible. These results are highly suggestive that the administration of GnRHa before and during chemotherapy might have minimized the gonadotoxic effects and increased the chance of spontaneous ovulation and successful conception and delivery.

More recently, a prospective, non-randomized study compared the rate of POF after SCT in adolescent and young women receiving GnRHa with gonadotoxic chemotherapy vs chemotherapy alone (94). The study found that GnRHa co-treatment may significantly decrease the POF rate from 82–33% in patients with lymphomas. Moreover, a recent single-center retrospective study on postmenarchal adolescent patients (median age 14, range 11 to 18) showed that GnRHa preserved ovarian function and fertility in adolescents treated for acute lymphoblastic leukemia, acute myeloid leukemia, Hodgkin lymphoma, or other cancers (100). On the last clinical visit, 29 patients (81%) had a regular menstrual cycle, three (8%) oligomenorrhea, and four (11%) amenorrhea. All the four patients with amenorrhea received HSCT. No differences were observed among patients' disease.

Even these trials confirmed the acceptable safety profile of GnRHa in this setting, with only frequent estrogen deprivation symptoms, reversible upon discontinuation, and bone metabolism alterations not significant for therapies <6 months.

Globally, these clinical data suggest that GnRHa may represents a very useful tool even in post-pubertal adolescents not only for reducing the risk of hypermenorrhea associated with hematologic malignancies or myelosuppressive chemotherapy, but also for preserving ovarian function and fertility, especially when the other established methods of FP (e.g. oocyte cryopreservation) cannot be performed, but also in combination with them in order to increase the odds of success for a specific patient.

At the moment, a phase II/III (NCT02856048), and two phase II (NCT04536467 and NCT03475758) randomized open-label trials including adolescents and pediatric patients are ongoing (**Table 3**).

THE REGULATORY PERSPECTIVE: OFF-LABEL USE

To date the three analogues (triptorelin, goserelin, leuprolide) are authorized for various therapeutic indications, such as cancer, endometriosis, uterine fibroids, and precocious puberty. Therefore, the prescription for preventing the risk of POF is typically an off-label use, defined as the use of a medicinal product "*for a medical purpose not in accordance with the authorized product information*" (109). European Union. Study on off-label use of medicinal products in the European Union. Available at: https://ec.europa.eu/health/sites/health/files. Off-label is not regulated at the European level, but specific national measures have been adopted (109, 110). In general, this use is not reimbursable excluding selected cases defined by law. For example, the *Recommandations Temporaires d'Utilisation* (RTU) provide coverage of recognized off-label treatment by France Health Insurance (111).

Moreover, the Italian national health system (NHS) reimburses an off-label use according to Law 648/1996 based on results from at least phase II trials (112). The inclusion into the 648/1996 list of reimbursable drugs ensures a nationwide access according to criteria for appropriate use and monitoring defined by the AIFA Scientific Committee in the light of clinical evidence.

From 2016 this Italian law allows to reimburse GnRHa for the preservation of ovarian function in pre-menopausal women at risk of premature and permanent menopause following chemotherapy treatment (29). The Italian regulatory authority defined the eligibility criteria, including age (>18 and <43) and lack of adequate alternative options. Thus, currently the use in the post-pubertal age is not approved and falls within the Italian Law 94/1998 (113), by which physicians can perform off-label prescriptions (not covered by the NHS) but in individual and exceptional cases.

This represents to date a limit for the treatment of this population that could be overcome if the eligibility criteria of Law 648/96 will be modified in order to include even pediatric patients.

Currently, to the best of our knowledge no other countries gave a nation-wide approval for this systematic off-label use.

TABLE 3 | Ongoing clinical trials (www.clinicaltrial.gov; update November 2020).

ID	Title	Trial design	Age range (years)	Number of estimated patients (disease)	Arms and interventions	Outcome measures	Follow-up duration
NCT02856048	Co-treatment With GnRH Analogs on the Ovarian Reserve in Young Women Treated With Alkylating Agents for Cancer (PRESOV Study), Sponsor Assistance Publique-Hôpitaux de Paris	Phase II/III randomized open-label	12–25	160 (Ewing Sarcoma, Osteosarcoma, Lymphoma)	1. Triptorelin 3 mg i.m. every 28 ± 3 days + Chemotherapy with alkylating agents at an intermediate ovarian toxicity risk* 2. Chemotherapy alone	Variation in AMH serum levels at 24 months; AFC on ultrasound at 24 months; delay of resumption of menses; AMH, FSH, estradiol levels monitoring; pregnancy rate at 3 years; GnRH-related AEs; change in BMD at 12 and 36 months	3 years
NCT04536467 (actual completion date June 1th 2020)	Prevention of Chemotherapy-Induced Ovarian Failure With Goserelin in Premenopausal Lymphoma Patients, Sponsor Beni-Suef University	Phase II randomized open-label	17–40	34 (Lymphoma)	1. Goserelin 3.6 mg s.c. 28 ± 3 days + standard chemotherapy 2. Standard chemotherapy alone	FSH and E2 levels at 6 months; overall response rate in lymphoma patients** at 6 months; GnRH-related AEs	6 months
NCT03475758	Goserelin for Ovarian Protection in Premenopausal Patients Receiving Cyclophosphamide, Sponsor Assiut University	Phase II randomized open-label	NR (Child and adult)	100 (Cancer patients)	1. Goserelin 3.6 mg s.c. every 4 weeks + cyclophosphamide containing chemotherapy 2. Cyclophosphamide containing chemotherapy alone	Rate of ovarian failure at 1 year (assessed by hormonal profile – FSH, LH, estradiol – every 6 months)	1 year

**Cyclophosphamide 6 g/m2, Ifosfamide 50 g/m2, Procarbazine 4 g/m2, Lomustine 350 mg/m2 or Melphalan 140 mg/m2 or a combination of these drugs; ** determined by tumor assessments from radiological tests (CT scan, MRI, Positron-emission tomography or physical examinations); AFC, Antral follicular count; AMH, Anti-Mullerian hormone; BMD, Bone Mass Density; FSH, Follicle-stimulating hormone; NR, not reported.*

CONCLUSION

Ovarian failure following chemotherapy represents an adverse event with an important impact on health and quality of life of survivors. Oocyte and tissue cryopreservation are the main options for fertility preservation. However, these techniques cannot be performed in all patients in all health-care facilities. Pharmacological treatment with GnRHa is a non-invasive and well-tolerated alternative, which can be offered to all subjects if cryopreservation is not feasible due to clinical or logistic issues. Moreover, combining several methods may increase the odds of success of fertility preservation in eligible patients. The available evidence demonstrates a significant advantage for the survivors who received the GnRHa in the long-term maintenance of ovarian function and preservation of fertility. Italy has officially recognized the off-label use of GnRHa in women at risk of premature and permanent menopause following chemotherapy. However, fertility preservation still represents an unmet medical need in adolescents, especially in those who cannot access to other therapeutic options.

AUTHOR CONTRIBUTIONS

SB and LG wrote the first draft of the manuscript. FD checked and revised the draft manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Dasatinib in the Management of Pediatric Patients With Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia

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Acute leukemia is the most common cancer in childhood; in particular, acute lymphoblastic leukemia (ALL) represents roughly up to 80% of all cases of acute leukemias in children. Survival of children with ALL has dramatically improved over the last few decades, and is now over 90% (versus 40% of adult patients) in developed countries, except for in infants (i.e., children < 1 year), where no significant improvement was registered. Philadelphia positive ALL (Ph+ALL) accounts for around 3% of cases of childhood ALL, its incidence increasing with patient's age. Before the era of tyrosine-kinase inhibitors (TKIs), pediatric Ph+ALL showed a worse prognosis in comparison to other forms of ALL, and was managed with intensive chemotherapy, followed, whenever possible, by allogenic hematopoietic stem cell transplantation (HSCT) in first morphological complete remission. TKIs have revolutionized the current clinical approach, which involves combinations of imatinib plus standard chemotherapy that can abrogate the negative prognostic impact conferred by the presence of BCR/ABL1 rearrangement, resulting in the probability of event-free survival (EFS) being significantly better than that recorded in the pre-TKI era. Long-term follow-up confirms these data, questioning the role of a real advantage offered by HSCT over intensive chemotherapy plus TKI in all Ph+ALL pediatric patients. Imatinib was the first generation TKI and the prototype of targeted therapy, but over the years second- (dasatinib, nilotinib, bosutinib) and third-generation (ponatinib) TKIs showed a capacity to overcome resistance to imatinib in Ph+ hematological neoplasms. Given the effectiveness of the first-in-class TKI, imatinib, also the second-generation TKI dasatinib was incorporated in the treatment regimens of Ph+ALL. In this manuscript, we will discuss the role of this drug in pediatric Ph+ ALL, analyzing the available data published to date.

Keywords: acute lymphoblastic leukemia, Philadelphia positive, dasatinib, children, tyrosine kinase inhibitor

INTRODUCTION

Through the application of reliable prognostic factors and risk-oriented treatment protocols, almost 85% of children with newly diagnosed acute lymphoblastic leukemia (ALL) can be cured today. The most common cause of treatment failure in pediatric ALL remains disease relapse, which occurs in approximately 15% of patients (1–8).

These improved patient outcomes in childhood ALL are due to many factors, including a better knowledge of the molecular lesions responsible for disease occurrence, monitoring of minimal residual disease (MRD), which represents a surrogate biomarker of leukemia cell sensitivity to chemotherapy, refined risk-adapted chemotherapy treatment and better results in patients given allogeneic hematopoietic stem cell transplantation (HSCT).

Despite these improvements in therapeutic management, ALL continues to impact the mortality rate of cancer in childhood. The outcome of refractory/relapsed ALL (r/rALL) remains, even nowadays, unsatisfactory and treatment must be diversified according to subsequent risk of treatment failure for children experiencing leukemia recurrence (8, 9). Innovative and flexible approaches need to be developed for timely treatment, along with more specific and effective drugs. However, at the same time, they need to be safely incorporated into the patient's treatment. Such agents are likely to provide the best opportunities for improving the long-term survival and of the patient's quality of life after ALL recurrence.

PH+ ALL

The discovery and definition of specific genetic abnormalities have increased knowledge of the biology of ALL. These have become the mainstay of clinical practice by providing relevant prognostic and predictive markers that influence treatment strategy and patient outcome. Although a wide range of genetic lesions have been discovered in childhood ALL, they are only partially relevant for prognosis (10). Response to treatment and prognosis of ALL can be strongly influenced by cytogenetic and molecular markers that can be associated with either good-risk or high-risk features. Among the cytogenetic/molecular abnormalities associated with a less favorable outcome, is the so-called Philadelphia chromosome, coming from t(9;22)(q34;q11), which is an encoded BCR-ABL1 fusion chimeric onco-protein with tyrosine kinase activity. The Philadelphia chromosome was first reported as the leading pathological alteration in chronic myeloid leukemia (CML). Then, in 1970, it was also found in ALL (11).

Ph+ ALL is usually associated with poor prognosis in both adulthood and childhood (12). It accounts for 3–4% of pediatric ALL cases (almost exclusively of B-cell origin) and about 25% of adult ALL cases (13). The incidence of Ph+ALL increases with age, being higher in adolescents than in younger children (the BCR-ABL1 fusion gene is detected in approximately 5-15% of adolescents). Before the era of TKIs, pediatric Ph+ ALL was associated with a dismal prognosis and was managed with intensive chemotherapy, followed, when possible, by HSCT in first remission (4). During recent decades, the availability of TKIs, in the context of CML, where the Philadelphia chromosome was first detected, had a dramatic impact on the management and prognosis of this disease (14). The revolutionary advancements in pharmacology provided by the advent of TKI led to the new concept of a targeted and "*personalized*" treatment of hematological neoplasms. ALL is not a unique disease, and its treatment strategy can be guided by the genetic and mutational landscape of the patient. The success of TKIs in CML has rapidly translated into attempts to treating other malignancies carrying the BCR-ABL1 fusion protein, including Ph+ ALL. Early published data have shown that in a Ph+ ALL pediatric population, imatinib combined with standard chemotherapy could reverse the negative impact on prognosis conferred by the presence of the BCR-ABL1 fusion transcript, resulting in a significant improvement in the probability of event-free survival (EFS) (15, 16). Long-term follow-up confirmed these data, questioning the role of HSCT in first complete remission as compared to strategies based on the combination of intensive chemotherapy and TKI in this category of patients (17). Imatinib was the first generation TKI and remains the prototype of targeted therapy, but, over the years, second- (dasatinib, nilotinib, bosutinib) and third-generation (ponatinib) TKIs have shown a capacity to overcome resistance to imatinib in Ph+ hematological neoplasms (18). Given the effectiveness of the first-in-class TKI, namely imatinib, the second-generation TKI dasatinib was also incorporated into treatment regimens for Ph+ ALL.

TARGETING PROTEIN KINASES

The human genome can encode for approximately 538 known protein kinases, whose activity maintains cellular function and cellular regulation through intracellular signaling pathways that are crucial for differentiation, survival, proliferation, metabolism, and cell-to-cell contact (19). Therefore, it is not surprising that protein kinases are one of the most relevant dysregulated molecules in human cancers, with several pathways that could lead to the proliferation of neoplastic cells in different types of hematologic and non-hematologic malignancies (19). Consequently, targeted therapy with small molecules and inhibitors against the activity of abnormal kinases is a leading method of treating hematological malignancies, and following imatinib, a first-generation TKI was approved for CML in 2001.

The Philadelphia chromosome results in the fusion gene BCR-ABL1 potentially existing in three principal isoforms. This is because it comes from different breakpoints on chromosome 22 in the BCR gene and encodes for three principal isoforms of aberrant protein kinases (namely, p190, p210, and p230) with distinct molecular mass (20). The frequency pattern of distribution of these isoforms is slightly different from CML to ALL and between adulthood and childhood (21, 22). In about 90% of cases of childhood Ph+ ALL, t(9;22) mostly occurs in the *minor breakpoint cluster region* and produces a constitutional activate tyrosin kinase

protein (of 190 kDa, p190 *BCR-ABL1*). The remaining cases are mainly represented by p210 isoforms (22).

Despite the different oncogenic activity in pre-clinical models between p190 and p210, there is no significant difference in terms of clinical outcome following chemotherapy in ALL patients harboring either of the two isoforms (23, 24). Regardless of the isoform, the chimeric BCR-ABL1 protein has direct effects on the oncogenic process by the ABL1 dysregulated and abnormal kinase activity that, in physiological conditions, is tightly controlled by a regulatory N-terminal region (25, 26). The chimeric BCR-ABL1 loses the regulatory region and, together with the boosting of BCR activity, physiological ABL1 functions are constitutionally activated. ABL1 is physiologically involved in a number of functions derived by interactions with other proteins. It is involved in the response of multiple extra and intracellular stimuli, playing a key role in cellular function, like cell-cycle or apoptosis (27). The final BCR-ABL1 mechanisms of transformation, as extensively studied in CML, are probably an altered cellular adhesion to stroma-cells and the matrix of bone marrow, triggering constitutively active mitogenic pathways together with inhibited apoptosis (28). As the majority of human neoplasms need multiple genetic steps to occur and determine the final neoplastic transformation, BCR-ABL1 is not the unique genetic alteration present in ALL and is not the unique neoplastic hit. However, given the effectiveness of TKIs in controlling the disease, BCR-ABL1 is potentially the major drive responsible for the abnormal proliferation of leukemia blasts in Ph+ALL. Therefore, targeting this dysregulated kinase activity represents a major treatment strategy for this leukemia.

Based on the mechanism of action, BCR-ABL1 kinase activity inhibition could be obtained through two major strategies: competitive inhibition and allosteric inhibition (29, 30). The first mechanism is provided by those ATP-competitive inhibitors, such as imatinib or dasatinib, whose binding site can be found in the catalytic cleft between the N-terminal lobe and C-terminal lobe kinase domain. These functional classes of molecules can be distinguished in type I and type II competitive inhibitors if they bind, respectively, to the activated/phosphorylated or inactivated/unphosphorylated conformation of kinase domain (31, 32). These inhibitors usually show scarce binding selectivity, which is particularly evident in type I over type II, providing inhibition or other kinases with consequently "off-target" side effects, like cardiac, pulmonary, gastrointestinal, and, especially in children, endocrine toxicity (33–36). The myristate binding domain or SH2-domain, are regulatory sites whose biological function is to quit an independent kinase activity, *via* different mechanisms (29). Therefore, the second type of inhibitor can bind to the regulatory sites that indirectly modulate the ATP-binding site conformation and activity in an allosteric fashion, providing highly selective kinase inhibition (37).

DASATINIB PHARMACOLOGY

When discussing the pharmacological properties of a TKI it is relevant to compare it with the prototype of this class of compound, namely imatinib. Dasatinib is an oral TKI, whose inhibitor activity is also directed to other protein kinases (38). It differs from its precursor imatinib in several ways, involving a potency of inhibition BCR/ABL wild-type expressing cells *in vitro* greater than 300-fold, different activity profile on the non-BCR/ABL kinases targeted, and the presence of other specific anti-leukemic properties involving MAPK or BCL2 pathways (39). Regarding its higher inhibiting potency compared to imatinib, it is believed that this is associated with its ability to bind both activated and non-activated conformation of the BCL-ABL kinase, as a type II competitive inhibitor, compared to imatinib, whose target is the activated isoform only (38, 39). Acting as a multiple protein kinases inhibitor and not only a BCR-ABL-directed molecule, dasatinib can offer multiple pharmacodynamic antineoplastic effects. Therefore, an anti-leukemic action is also provided by blockage of the Stat-5 downstream pathway of BCR-ABL and SRC kinases family, which could reduce neoplastic proliferation and stimulate apoptosis (40). It could also interfere with p38 Map kinase of the MAPK family, which is demonstrated to be essential for the anti-leukemic effect of dasatinib (41).

In vitro data shows that dasatinib contributes to the anti-leukemic effect of imatinib-resistant neoplastic cells, even if some point mutations in BCR/ABL confer several degrees of resistance to dasatinib, with the maximum resistance displayed by T315I mutation, as also to the majority of available TKIs (42, 43). Off-target effects involving other kinases and targets are recognized as being responsible for some adverse events (AE) of dasatinib administration. The activity of hematopoietic cells is affected by the direct interaction of dasatinib with the BTK and TEC kinases, resulting in an impaired B- and T-cell development effect (44, 45). Pleural effusion, with characteristic lymphocyte-rich fluid, is a relatively common (20-35% of patients) AE reported with dasatinib treatment and is also probably caused by a specific immune-mediated off-target pharmacodynamic effect involving the PDGFR-beta pathway (46, 47).

Dasatinib has been shown to penetrate the central nervous system (CNS) at considerably higher levels, as confirmed also by more recent studies (48, 49).

DASATINIB IN PEDIATRIC PH + ALL— CLINICAL EXPERIENCES

Phase I Trial

Zwaan et al. conducted a phase I trial in pediatric patients affected by imatinib-resistant or intolerant Ph+ CML, relapsed and refractory Ph+ ALL and relapsed Ph+ AML (50), in which dasatinib was administered in once-daily dose-escalation (starting from 60 until 120 mg/m2).

The efficacy and safety were comparable to adult results, with no response in Ph-negative relapsed/refractory ALL or AML. 60 mg/m^2 and 80 mg/m^2 once-daily were selected for phase II trials in Ph+ ALL.

Phase II Trial—COG AALL0622

Slayton et al. conducted a phase II trial (COG AALL0622) in newly diagnosed children with Ph+ ALL, with dasatinib replacing imatinib on day 15 of induction. It was administered in combination with the same chemotherapy approach used in COG AALL0031 (51). Endpoints were safety and feasibility in 1-30-year-old patients. HSCT was recommended in high-risk/slow responder patients, and also in patients with a matched-family-donor independent from response. Standard-risk patients lacking an HLA-matched donor were managed with a combination of chemotherapy and dasatinib for 120 additional weeks, while CNS positive patients underwent cranial irradiation. Dasatinib plus chemotherapy showed good tolerability and outcomes similar to imatinib in COG AALL0031 (5-year OS 86% ± 5% overall, 87% ± 5% for high-risk patients); 5-year rate (± SD) of CNS relapse was 15% ± 6%. These findings confirm data obtained using imatinib and chemotherapy, with the recommendation to reserve HSCT only to slow responders, suggesting IKZF1 as a new biomarker whose potential role should be further investigated.

COG AALL1131

The COG Trial (AALL1131) explored the role of dasatinib in newly diagnosed, high-risk Ph-like B-ALL, harboring ABL-class lesions (52). The authors identified new rearrangement partners which could be potential targets. This needs to be better and further explored in new trials aimed at detecting specific alterations in this particular subset.

Phase II Trial—CA180-372

CA180-372 was an international phase 2 clinical trial, aiming to explore the combination of continuous daily dasatinib (daily dose of 60 mg/m^2 from day 15 of induction) plus EsPhALL chemotherapy in pediatric Ph+ ALL (53). Minimal residual disease (MRD) was evaluated at day 78, at the end of phase 1b, by several methods (Ig/TCR PCR, flow cytometry, and BCR - ABL1 RT-PCR). Patients who remained MRD positive at any detectable level after three additional high-risk chemotherapy blocks were candidates to receive HSCT in first complete remission (CR1), while dasatinib maintenance was optional. The other patients received a combination of dasatinib plus chemotherapy for 2 years, with cranial irradiation limited to CNS3 patients. This combination was safe and effective (in terms of 3-year EFS) in pediatric Ph+ ALL patients, with 14% who underwent HSCT in CR1, versus 80% in the EsPhALL trial.

Total XVI Study

Jeha et al. designed the Total XVI Study (54, 55), comparing the outcome of Ph+ ALL in the pre-TKIs era versus TKIs-based treatments. TKIs (including dasatinib) were administered, starting from day +22 of induction therapy during all treatment phases, showing significant results in terms of MRD if compared with chemotherapy alone, in terms of 5-year EFS (68.6 ± 19.2% and 31.6 ± 9.9%, respectively (P = .022), confirming that the administration of TKIs in the early phases of treatment improves the outcome of pediatric Ph+ ALL.

DASATINIB VERSUS IMATINIB IN THE TREATMENT OF PEDIATRIC PHILADELPHIA CHROMOSOME–POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA—A RANDOMIZED CLINICAL TRIAL

Shen et al. (49), designed an open-label, phase 3, randomized clinical trial, including 225 patients from 20 hospitals in China. The trial examined whether dasatinib, at a daily dosage of 80 mg/m2, is more effective than the first-generation inhibitor imatinib mesylate, at a daily dosage of 300 mg/m2. It aimed to improve event-free survival in children with Philadelphia chromosome–positive ALL who had received intensive chemotherapy without prophylactic cranial irradiation (the secondary outcomes were relapse, death due to toxic effects, and overall survival).

The 4-year event-free survival and overall survival rates were 71.0% (95% CI, 56.2%-89.6%) and 88.4% (95% CI, 81.3%-96.1%), respectively, in the dasatinib group and 48.9% (95% CI, 32.0%-74.5%; P = .005, log-rank test) and 69.2% (95% CI, 55.6%-86.2%; P = .04, log-rank test), respectively, in the imatinib group. The 4-year cumulative risk of any relapse was 19.8% (95% CI, 4.2%-35.4%) in the dasatinib group and 34.4% (95% CI, 15.6%-53.2%) in the imatinib group (P = .01, Gray test), whereas the 4-year cumulative risk of an isolated central nervous system relapse was 2.7% (95% CI, 0.0%-8.1%), excellent control of central nervous system leukemia without the use of prophylactic cranial irradiation, in the dasatinib group and 8.4% (95% CI, 1.2%-15.6%) in the imatinib group (P = .06, Gray test). There were no significant differences in the frequency of severe toxic effects between the 2 treatment groups.

To date, this is the first clinical trial comparing the use of dasatinib versus imatinib in pediatric Ph-positive ALL settings, encouraging a switch in future studies.

CONSENSUS PAPER

In a consensus paper, major experts in Ph+ ALL agree that HSCT remains the standard of care in adult patients. It outlined improved outcomes, thanks to the use of TKIs in frontline therapy, but with many patients still relapsing after the allograft. TKIs-based maintenance post-HSCT can reduce relapse risk and should be considered a valuable option (56). Future studies addressing the same issue in a pediatric population are needed, although some clinical experiences support the positive role of the drug in this clinical setting (57).

DASATINIB AS A BRIDGE TO THE SECOND ALLOGRAFT IN POST HSCT RELAPSED PH+ALL

A case report showed a Ph+ALL patient with early relapse after first HSCT, who was given dasatinib single agent treatment,

achieving complete molecular remission, which persisted for 12 months after the second HSCT, with acceptable tolerability (58).

Another clinical case explored dasatinib in early relapsed Ph + ALL post HSCT. In this patient, after complete molecular response, dasatinib was used as a bridge to a second successful transplant, also showing a very good safety profile (59).

NOVEL COMBINATIONS: THE NEXT FUTURE OF DASATINIB-BASED TREATMENT

A novel approach could be the combination of dasatinib plus ABT-199/venetoclax, which is a BCL2 (protein B-cell lymphoma 2) inhibitor. It showed improved antileukemic efficacy with equivalent tolerability if compared to either of the single agents in Ph+ ALL xenografted immunodeficient mice (60). This combination showed high synergism *in vitro*, with the decrease of cell viability and the induction of apoptosis in Ph + ALL, and, thanks to multikinase inhibition, it was shown to add the advantage of inducing Lck/Yes novel tyrosine kinase (LYN)-mediated proapoptotic BCL-2-like protein 11 (BIM) expression and inhibiting up-regulation of antiapoptotic myeloid cell leukemia 1 (MCL-1), potentially overcoming venetoclax resistance. These data are encouraging and clinical trials exploring this interesting combination are planned for the future.

CONCLUSIONS

In recent years there has been important progress in the management of pediatric ALL, thanks to risk-adapted protocols and CNS prophylaxis, while the prognosis for those with Ph+ALL remained unfavorable until the beginning of the TKIs era.

The second-generation TKI dasatinib, an oral inhibitor of chimeric BCR-ABL oncogenic kinase with multi-inhibitor activity, showed improved outcomes if used in combination with the standard chemotherapy approach. Moreover, in a post-HSCT setting, it could have potential benefits in the maintenance of this condition, but more solid data and further studies are required.

Preliminary data about the combination with other molecules, such as the BCL2-inhibitor venetoclax, are promising and confirm the need for further exploration of these combinations, which could form the backbone of new risk-adapted/MRD-driven clinical trials in relapsed Ph+ALL.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to: the conception and design of the study, or acquisition of data, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and approved the final submitted version.

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Management of Oral Mucositis in Children With Malignant Solid Tumors

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Introduction: In recent years, the use of intensive regimens for the treatment of pediatric cancer has led to a marked improvement in patient survival. However, these treatments are associated with an increase in toxic effects. Among these side effects, mucositis (inflammation of the oral cavity) significantly affect the success of treatment. The aim of this study was to assess the prevalence of mucositis in a pediatric population with solid tumor and undergoing chemotherapy, identify the risk factors that influence its occurrence, and verify the usefulness of pain rating scales.

Methods: We registered episodes of mucositis which occurred in a sample of 84 consecutive children with solid tumors between 1 January, 2012 and 30 April, 2018. The World Health Organization (WHO) oral mucositis grading scale and the modified Wong–Baker FACES Pain Rating Scale (WBS) were used to assess the severity of each episode. Moreover, data on the treatments used and blood count results were collected.

Results: The prevalence of mucositis in our population was 50%, without statistically significant difference according to sex and a higher prevalence observed in patients aged >10 years. The presence of neutropenia, higher number of cycles of chemotherapy, and co-existence of lymphomas and sarcomas were identified as factors favoring the occurrence of mucositis. The WBS showed results superimposed on the WHO oral mucositis grading scale in choosing the intensity and duration of mucositis treatment.

Conclusion: Oral mucositis is a common complication of chemotherapy against childhood malignancies. The WHO oral mucositis scale is a valuable tool for assessing its severity in pediatric patients. Furthermore, WBS can be used as an assessment tool to establish the therapy to be adopted for patients in whom direct evaluation of the oral cavity is not possible.

Keywords: children, cancer, mucositis, chemotherapy, pain

INTRODUCTION

Pediatric cancers are rare diseases, although their incidence has increased in recent years. However, the cumulative overall survival is increased due to the adoption of international cooperative treatment regimens, which permit the combination of surgery, radiotherapy and chemotherapy. In parallel with the increase in survival, increased toxicity has been observed both at the hematological and extra-hematological levels (1–5).

Along with nausea and vomiting (6), mucositis is one of the most frequent side effects associated with the treatment of pediatric cancers; it is an inflammation of the mucosa of the oral cavity with multiple etiologies causing pain and inability to eat. Lack of control and inadequate prevention of mucositis can lead to a significant decline in patient quality of life (i.e., pain, difficulty feeding, and malnutrition) (7–9).

The incidence rate of oral mucositis ranges from 52% to 100% of patients receiving high-dose chemotherapy. If not managed with adequate measures, mucositis represents an important limiting factor of chemotherapy and may worsen patient prognosis and compliance (10–12).

Appropriate oral hygiene represents the main intervention in children receiving chemotherapy (13, 14). In addition, in the presence of mucositis, a systematic pain assessment allows the selection of appropriate treatment and reduces the rate of treatment-related side effects. Although there are numerous publications on the prevention and treatment of oral mucositis, a specific standard treatment protocol for children with cancer is currently unavailable (15).

The main objective of this study was to evaluate the prevalence of mucositis in a pediatric population, identify the risk factors that influence its occurrence, and verify the usefulness of pain rating scales. The secondary objective of the study was to verify the effectiveness of the pain treatment routinely adopted at our center.

METHODS

Our study assessed 84 consecutive children with solid tumors treated in the Paediatric Oncology Unit of Agostino Gemelli Hospital (Rome, Italy) between 1 January 2012 and 30 April 2018. Inclusion criteria were: (a) age 4–18 years; (b) diagnosis of solid tumor; (c) administration of at least one course of chemotherapy during the study period; (d) no concomitant radiation therapy; and (e) no surgery on the head region, face, and digestive system.

Of 84 patients examined, 33 were affected by central nervous system tumors, 23 patients by sarcomas, 11 patients by lymphomas and 17 patients by other solid tumors (mainly neuroblastomas, nephroblastomas, and retinoblastomas).

Patients with leukemia were not included in the study due to the different type of treatment they undergo. Patients with solid tumors receive chemotherapy courses every three to four weeks while patients with leukemia receive chemotherapy treatments with shorter timescales. For this reason, in our study we did not consider these two categories of patients to be comparable.

All patients were given an oral hygiene protocol based on the use of a soft toothbrush for patients with a platelet count <50,000 cells/ml, or a sponge brush or a wet gauze for patients with a platelet count <20,000 cells/ml, and prophylactic oral rinsing/swabbing with alcohol-free 0.2% chlorhexidine oral rinse for four times a day (16).

If mucositis was diagnosed, the World Health Organization (WHO) oral mucositis grading scale was adopted to evaluate its severity (17) (**Table 1**).

In addition, all patients underwent evaluation using a Pain Rating Scale. We adopted the modified Wong–Baker FACES Pain Rating Scale (WBS) with a score between 0 and 5 (18). This evaluation was performed thrice daily.

In order to be able to compare the two scales, we excluded patients under the age of 4 due to the possible difficulty in carrying out a complete evaluation of the oral cavity as required by the WHO oral mucositis grading scale.

All children who were admitted and had received chemotherapy within the past 14 days were considered neutropenic when the neutrophil count was <500 cells/ml.

Based on the intensity of pain and duration of symptoms, pain relief treatment was initiated with pethidine through continuous intravenous infusion at a dosage of 2 mg/kg/day. Clonazepam was orally administered in combination (1 drop/10 kg/day) to prevent the risk of seizures associated with pethidine. If treatment with pethidine was ineffective against pain, fentanyl was administered intravenously through continuous infusion at the initial dosage of 0.5 μg/kg/h. All patients underwent multiparametric monitoring during treatment. Finally, all patients with evident inability to eat underwent intravenous parenteral nutrition.

Data are presented as the mean and standard deviation (SD). The Kruskal–Wallis test was used for comparison of values between groups. The chi-squared test was used for comparison of proportions. The Spearmen's linear correlation coefficient (r) was used to study the relationship between continuous variables. A p-value <0.05 denoted statistically significant differences.

The patients evaluated were not subjected to treatments or procedures other than those required by the mucositis management protocols approved by our facility. Prior to the data analysis, the caregivers provided signed consent forms after being informed about the aim of the project as foreseen by the Italian Law on Privacy and Safeguarding of Sensitive Data (D. Lgs n196,2003). In the text there are no data that can be traced back specifically to any patient.

RESULTS

84 patients were selected (mean age: 13.1 years [SD: 5.6]; 43 males [51%]; 41 females [49%]). Of those, 42 patients had at least

TABLE 1 | The WHO oral mucositis grading scale (17).

Grade	Description
0 (none)	None
I (mild)	Oral sorenes,erythema
II (moderate)	Oral erythema, ulcers, solid diet tolerated
III (severe)	Oral ulcers, liquid diet only
IV (life-threatening)	Oral alimentation impossible

one episode of mucositis, that is 50% of the population, {mean age: 13.6 years [SD: 5.2]; 19 females (45.2%); 23 males (54.8%); 33 (79%) and nine (21%) patients were aged >10 and <10 years, respectively}. There was no difference in the incidence of mucositis in relation to sex ($p = 0.9$), while the incidence was higher in patients aged >10 years ($p < 0.001$). However, a relationship was not identified between the number of episodes and age of patients ($r = 0.15$; $p = 0.18$).

The number of episodes of mucositis caused by chemotherapy varied in the study population. Of the 42 patients, 16 had one episode (38.1%), seven patients had two episodes (16.7%), seven patients had three episodes (16.7%), four patients had four episodes (9.5%), two patients had five episodes (4.8%), four patients had six episodes (9.5%), and only two patients had seven total events (4.8%) throughout the duration of the drug treatment. We identified an increasing linear relationship between the number of episodes of mucositis and the number of cycles of chemotherapy administered ($r = 0.52$; $p < 0.01$) (**Figure 1**).

The time of onset of mucositis was measured in days, starting on day 0 as the first day of chemotherapy. The occurrence of mucositis has been observed from a minimum of 5 days to a maximum of 17 days (mean: 10.4 days [SD: 3.6]). The duration of mucositis ranged 2–20 days (mean: 7.3 days [SD: 4.89]).

Patients were stratified according to the diagnosis into four groups: (1) patients with central nervous system tumors; (2) patients with sarcomas; (3) patients with lymphomas; and (4) patients with solid tumors, mainly neuroblastomas, nephroblastomas, and retinoblastomas. Mucositis events were also analyzed by following their distribution among the same subgroups used for the classification of patients. **Table 2** summarizes the characteristics of patients and episodes of mucositis stratified by the types of tumors.

Based on pain assessment using the WBS, we registered the following: 26 episodes, score 1 (22.6%); five episodes, score 2 (4.4%); 26 episodes, score 3 (22.6%); 25 episodes, score 4 (21.7%); and 33 episodes, score 5 (28.7%).

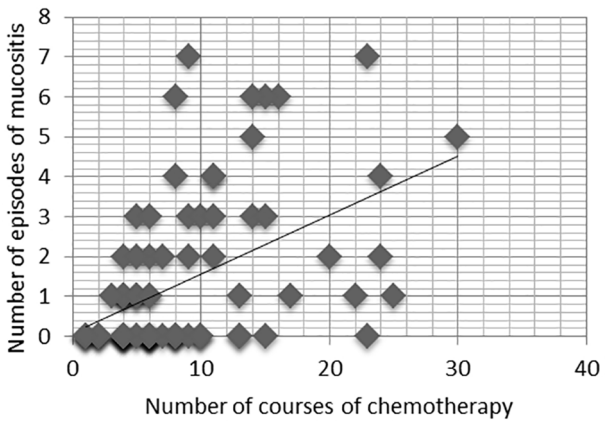

FIGURE 1 | Relationship between the number of courses of chemotherapy and the number of episodes of mucositis, r=0.52, p< 0.01.

TABLE 2 | Characteristics of the children stratified by tumor's type.

	Number of patients	Average age (years)	Number of CT cycles	Average number of CT cycles/ patients
CNS Tumor	33	11,8	169	5
Sarcomas	23	15,1	334	13
Lymphomas	11	18	110	6
Other	17	7,7	162	6
p		**0,01**		**0,01**
	Number of patients with mucositis	**Total number of episode of mucositis observed**	**Average number of CT cycles with mucositis /patients**	**Percentage of cycles of CT with mucositis (%)**
CNS Tumor	6	10	1,7	6
Sarcomas	18	56	3	17
Lymphomas	6	21	3,5	19
Other	12	28	2,3	17
p			**0,01**	**0,01**
	Number of patients with mucositis and neutropenia	**Total number of CT cycles with mucositis and neutropenia**	**Average number of CT cycles with mucositis and neutropenia/patients**	**Percentage of cycles of CT with mucositis and neutropenia (%)**
CNS Tumor	6	9	1,5	5,5
Sarcomas	15	45	3	13,5
Lymphomas	6	16	2,7	14,5
Other	11	18	1,6	11,1
p			**0,01**	**0,01**
	Number of patients without any episodes of mucositis	**Total number of CT cycles without mucositis**	**Average number of CT cycles without mucositis /patients**	**Percentage of cycles of CT without mucositis (%)**
CNS Tumor	27	159	4,8	94
Sarcomas	5	278	12,1	83
Lymphomas	5	89	4,9	81
Other	5	134	7,9	83
p			**0,01**	**0,01**

The episodes of mucositis were classified according to the WHO oral mucositis grading scale: 27 episodes, grade I (23.5%); 28 episodes, grade II (24.4%); 25 episodes, grade III (21.7%); and 35 episodes, grade IV (30.4%). **Table 3** summarizes the characteristics of episodes of mucositis grouped according to the score obtained using the WBS and their class in the WHO oral mucositis grading scale.

Analysis of the relationship between the pain expressed by patients through the WBS and the objectivity found through the WHO oral mucositis grading scale showed the following: for WHO I, 24 episodes of 27 received a score equal to 1 of the WBS (88.89%), one episode a score of 2, and two episodes a score of 5; for WHO II, 15 of 28 episodes (53.57%) received a score of 3, six episodes a score of 4, four episodes a score of 2, and only one episode a score of 5; for WHO III, we observed a fair distribution between WBS 3 and 4, with 11 (44%) and 14 (56%) cases of 25 respectively; for WHO IV 30 of 35 cases (85.7%) had a score of 5, while the remaining five had a score of 4 (14.3%). **Figure 2** illustrates the preceding data.

Furthermore, we have evaluated the relationship between neutropenia (<500 cells/ml) and the onset of oral mucositis. Therefore, we analyzed the number of episodes of neutropenia affecting the 84 patients during the observation period (i.e., during the 775 total chemotherapy cycles administered) and the number of episodes of neutropenia associated with mucositis. The odds ratio was 20.3 ($p < 0.01$). A total of 78 episodes of mucositis (68%) were linked to a neutrophil count <500 cells/ml. Hence, confirmation of neutropenia is a significant risk factor for the development of this complication. There was no relationship between the neutrophil count and severity of pain assessed by the WBS or WHO oral mucositis grading scale; in fact the averages of the minimum value of neutrophils observed during the episodes of mucositis were not statistically different into the WBS class and WHO grade (**Table 2**, **Figure 3**).

In 89 episodes of mucositis (77.4%), a pain-relieving pharmacological treatment was required in addition to the mouth rinses for seven, 22, 25, and 35 cases of WHO grades I, II, III, and IV mucositis, respectively.

Pain relief was achieved in 79 episodes (68.7%) following the intravenous administration of pethidine for seven, 21, 22, and 29 cases of WHO grades I, II, III, and IV, respectively.

In 10 episodes (8.7%), fentanyl was used to support oral rinses: four and six cases of WHO grades III and IV, respectively.

Finally, 26 episodes (22.6%) did not require any additional drug supplement to the oral rinses: 20 and six cases of WHO grades I and II, respectively.

The duration of drug treatment was longer for children with WHO grades III and IV (**Table 2**). This difference was statistically significant ($p = 0.04$). Total parenteral nutrition was prescribed for 26 episodes of 115 (22.6%). We did not observe correlations between neutrophil count and the duration of drug treatment ($r = -0.02$; $p = 0.87$).

DISCUSSION

In recent years, there has been a noticeable improvement in terms of survival for children with cancer. This was achieved owing to the intensification of medical treatments which, however, led to a parallel increase in the rate of side effects (1–3, 19).

Mucositis represents one of the most frequent side effects in children receiving chemotherapy with an incidence ranging 52–100% of the cases described in the literature (17). This finding was

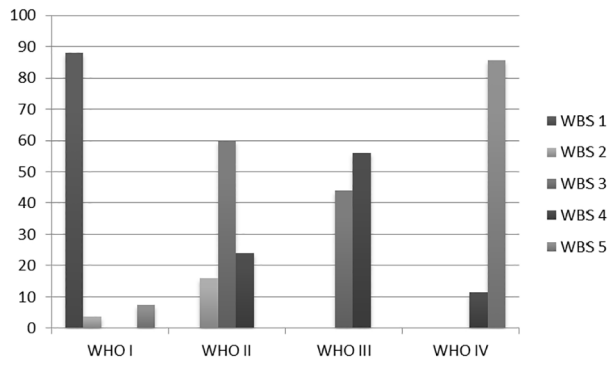

FIGURE 2 | Relationship between the pain expressed by patients through the WBS and WHO oral mucositis grading scale.

TABLE 3 | Characteristics of the mucositis episodes.

WBS	Number of episodes	Average minimum number of neutrophils /episodes (cells/ml)	Percentages of episodes that needed treatment (%)	Average duration of treatment /episodes (days)
1	26	155	15	6
2	5	135	80	5,5
3	26	215	61,5	6
4	25	200	68	8
5	33	160	100	9
p		0,76	**<0,01**	0,07
WHO oral mucositis grading scale	**Number of episodes**	**Average number of neutrophils /episodes (cells/ml)**	**Percentages of episodes that needed treatment (%)**	**Average duration of treatment /episodes (days)**
I	27	110	33,3	5,5
II	28	220	78,6	6
III	25	200	100	7
IV	35	160	100	9
p		0,28	**<0,01**	**0,04**

In the first part of the table the episodes are grouped according to the score obtained in the WBS. In the second part the episodes of mucositis are grouped according to the class they belong to the WHO oral mucositis grading scale.

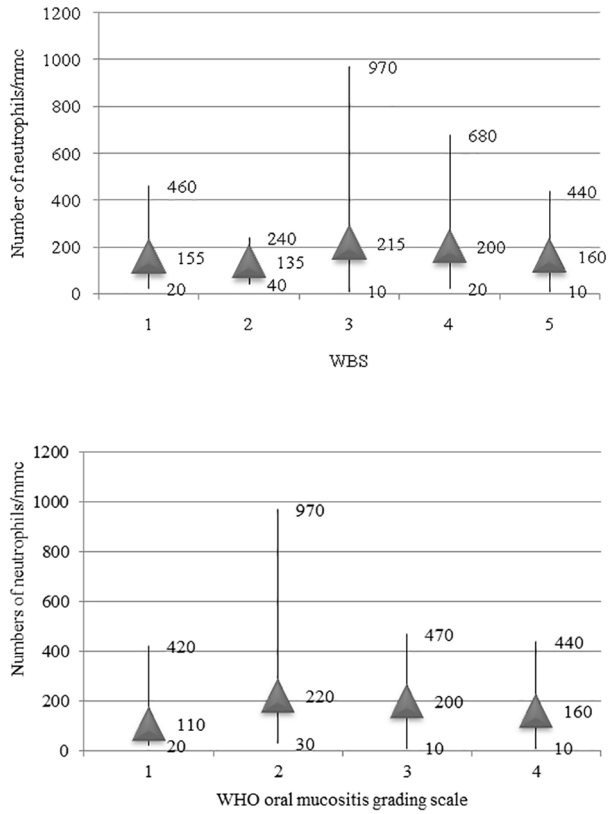

FIGURE 3 | Relationship between the minimum number of neutrophils observed during the episode of mucositis and the grade of mucositis according to WBS and WHO oral mucositis scale.

confirmed in our population, in which we diagnosed mucositis in 50% of the patients.

Recent studies have reported a difference in the prevalence of mucositis in relation to sex. However, these data are not homogeneous as some investigators reported a female predominance (20), whereas others noted a male predominance (21). In our population, there was no difference in relation to sex observed; hence, it appears that sex is not a factor influencing the occurrence of mucositis.

Although a correlation between age and the number of episodes of mucositis was not detected in our patients (21), the incidence of mucositis was significantly higher in children aged >10 years. We hypothesized that this result is linked to the lower ability for oral mucosa renewal in older children (22) and the less attention paid by adolescents to oral hygiene.

On average, mucositis episodes occurred 10.4 days after the initiation of chemotherapy and lasted 7.3 days, as recently demonstrated also by Hurrell et al. (23).

According to the diagnosis, patients were stratified into four groups: patients with central nervous system tumors; patients with sarcomas; patients with lymphomas; and patients with the remaining solid tumors, which mainly included neuroblastomas, nephroblastomas, and retinoblastomas. We observed a greater number of episodes of mucositis in patients with sarcomas or lymphomas. This relationship may be linked to the correlation between the number of cycles of chemotherapy performed and episodes of mucositis observed ($r = 0.52$; $p < 0.01$), as well as the different antineoplastic agents used in the respective treatment protocols. The use of polychemotherapy did not allow us to identify the drugs most frequently implicated in the onset of mucositis. However, methotrexate and anthracyclines are frequently utilized for the treatment of lymphomas and sarcomas. These two chemotherapeutic agents are among the drugs with the greatest risk of causing mucositis. This was confirmed by the higher incidence of mucositis in patients receiving chemotherapy for leukemia, for whom treatment with anthracyclines and methotrexate is essential, as observed in previous studies (19–24).

Furthermore, lymphomas and sarcomas are the two types of cancer in which we observed the highest prevalence of neutropenia-associated mucositis. Neutropenia is an established risk factor for the development of mucositis (25, 26). This has also been confirmed by our investigation, which showed an odds ratio equal to 20.3 ($p < 0.01$) for neutropenia. The presence of neutropenia, linked to more intensive therapeutic treatments, is certainly an important factor influencing the higher prevalence of mucositis recorded in the group of patients with sarcomas and lymphomas. However, in our study we included only patients undergone to standard chemotherapy and not to high-dose chemotherapy. For this reason we cannot be sure that the degree of neutropenia will not affect the severity and duration of mucositis in the case of high-dose chemotherapy with more intense neutropenia.

By adopting the WHO oral mucositis grading scale, we observed that all episodes of mucositis classified as more serious (classes III and IV) required pain relief treatment through the intravenous route. Furthermore, the duration of treatment for these episodes was significantly longer than that for less serious episodes (classes I and II) ($p = 0.04$). Therefore, use of the WHO oral mucositis grading scale is effective in adequately assessing the severity of episodes of mucositis in children (27).

The WHO oral mucositis grading scale provides an in-depth assessment of the oral cavity. However, in pediatric patients, this is not always possible due to poor compliance. Therefore, we attempted to analyze the possible correspondence between the WHO oral mucositis grading scale, an objective and integrative assessment scale of signs and symptoms, with a pain assessment scale widely used and validated in children, such as the WBS (19). The WBS is easy to apply in pediatric patients, as it allows the collection of data relating to the discomfort experienced by the patient without the need to resort to an objective evaluation, which is not always obtainable in this group. Moreover, it can be easily repeated several times during the same day to adequately modulate therapy. Similar to the WHO oral mucositis grading scale, the episodes described as more serious directly by the patient were those that required intravenous drug treatment. Furthermore, we observed an almost total overlap between the degree of pain described by the patients and the objectivity found during the evaluations performed using the WHO oral mucositis grading scale. This allows us to conclude that, in patients in whom an objective assessment of the degree of mucositis is not possible, application of the WBS may be sufficient in guiding the choice of intravenous treatment.

As previously mentioned, the presence of neutropenia represents a risk factor for the development of mucositis. However, we observed that the degree of neutropenia does not affect the severity of the mucositis episode and does not influence the duration of treatment ($r = -0.02$; $p = 0.87$).

Although the data in the literature are often discordant and not unequivocal in interpretation, the importance of adequate oral hygiene as a protective factor for the development of mucositis appears to be definitive. The use of specific oral hygiene protocols for prophylactic purposes associated with a continuous education intervention performed by health personnel, though not preventing the onset of mucositis, has proved to be fundamental in reducing its duration and severity (28).

In the study population, the oral hygiene protocol was respected in almost 100% of cases, which allowed for a prevalence of mucositis equal to 50%, slightly lower than the rates previously described in the literature (12).

In 89 episodes of mucositis (77.4%), a pain-relieving pharmacological treatment was required in addition to the mouth rinses; in 79 episodes (68.7%), intravenous administration of pethidine was sufficient, while in 10 episodes (8.7%), patients needed more intensive drug treatment with intravenous fentanyl through continuous infusion. These 10 episodes belonged to classes III and IV of the WHO oral mucositis grading scale, confirming the effectiveness of this evaluation system in predicting the intensity of the treatment to be adopted.

Therefore, therapy with intravenous pethidine was effective in 68.7% of cases, and only 8.7% necessitated more intense drug treatment, such as intravenous fentanyl. During patient observation, therapy with pethidine was absolutely safe and did not lead to any side effects. Thus, patients perfectly tolerated and responded promptly to the treatment. This confirms the findings of a previous study conducted by Oudot et al. (29), which revealed that the use of pethidine demonstrated the same efficacy as that of major opioid drugs, but with fewer side effects.

Only 26 cases of the 115 analyzed (22.6%) required parenteral feeding. In fact, the severity and duration of mucositis were such that the use of pethidine, fentanyl, and oral rinses was insufficient to improve the functionality of the oral cavity and allow the patient to quickly recover the ability to independently drink and eat.

CONCLUSION

Oral mucositis is a common complication of chemotherapy for childhood malignancies, which has an important influence on patient quality of life and compliance to treatment. In our study, 50% of the patients experienced at least one episode of mucositis during the treatment period. Moreover, the factors that affected its occurrence are the presence of neutropenia, the number of cycles of chemotherapy performed, and the type of tumor.

The WHO oral mucositis scale is a valuable tool for assessing the severity of mucositis in pediatric patients and the WBS can be used as an assessment tool for establishing the therapy to be adopted in patients in whom direct evaluation of the severity of mucositis is not possible.

The treatment currently used in our clinical practice is effective and safe, without significant adverse events recorded.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Prior to their participation, the caregivers provided signed consent forms after being informed about the aim of the project as foreseen by the Italian Law on Privacy and the Safeguarding of Sensitive Data (D.Lgs n196, 2003). The project was performed in accordance with the principles of the Declaration of Helsinki. Approval by the ethics committee was not necessary for a retrospective observational study on clinical practice data.

AUTHOR CONTRIBUTIONS

Conceptualization, ARu, ARo, and GA. Methodology, ARu and PM. Writing—original draft preparation, ARo, MC, and ST. Writing—review and editing, SM and AD. Supervision, ARu. All authors contributed to the article and approved the submitted version.

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Use of Chronic Prescription Medications and Prevalence of Polypharmacy in Survivors of Childhood Cancer

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Background: As survivors of childhood cancer age, development of cancer treatment-related chronic health conditions often occur. This study aimed to describe the pattern of chronic prescription medication use and identify factors associated with polypharmacy among survivors of childhood cancer.

Methods: This was a retrospective study conducted at the pediatric oncology long-term follow-up clinic in Hong Kong. Eligible subjects included survivors who were (1) diagnosed with cancer before 18 years old, (2) were at least 3 years post-cancer diagnosis and had completed treatment for at least 30 days, and (3) receiving long-term follow-up care at the study site between 2015 and 2018. Dispensing records of eligible survivors were reviewed to identify medications taken daily for ≥30 days or used on an "as needed" basis for ≥6 months cumulatively within the past 12-month period. Polypharmacy was defined as the concurrent use of ≥5 chronic medications. Multivariable log-binomial modeling was conducted to identify treatment and clinical factors associated with medication use pattern and polypharmacy.

Results: This study included 625 survivors (mean current age = 17.9 years, standard deviation [SD] = 7.2 years) who were 9.2 [5.2] years post-treatment. Approximately one-third (n = 219, 35.0%) of survivors were prescribed at least one chronic medication. Frequently prescribed medication classes include systemic antihistamines (26.5%), sex hormones (19.2%), and thyroid replacement therapy (16.0%). Overall prevalence of polypharmacy was 5.3% (n = 33). A higher rate of polypharmacy was found in survivors of CNS tumors (13.6%) than in survivors of hematological malignancies (4.3%) and other solid tumors (5.3%) ($P = .0051$). Higher medication burden was also observed in survivors who had undergone cranial radiation (RR = 6.31; 95% CI = 2.75–14.49) or hematopoietic stem-cell transplantation (HSCT) (RR = 3.53; 95% CI = 1.59–7.83).

Conclusion: Although polypharmacy was observed in a minority of included survivors of childhood cancer, chronic medication use was common. Special attention should be paid to survivors of CNS tumors and survivors who have undergone HSCT or cranial radiation. These individuals should be monitored closely for drug–drug interactions and adverse health outcomes that may result from multiple chronic medications, particularly during hospitalization in an acute care setting.

Keywords: childhood cancer survivor, pediatric oncology, chronic medication prescriptions, polypharmacy, medication utilization

INTRODUCTION

Advances in medical knowledge and treatment of cancer have improved the 5-year survival rate of patients with childhood cancer from 50% three decades ago to more than 80% at present (1, 2). However, cancer survivorship comes with a cost of developing a myriad of chronic health conditions as the late adverse effects of cancer treatment (3–7). It is reported that more than three-quarters of childhood cancer survivors suffer from at least one late effect within 35 years after the end of treatment (4). Survivors of childhood cancer are also eight times more likely to develop a severe or life-threatening chronic condition such as cardiomyopathy, premature gonadal failure, metabolic syndrome, and neurocognitive dysfunction (3).

Management of these late effects may require lifelong medications. Among adult cancer survivors, the reported prevalence of polypharmacy, i.e., the concurrent use of multiple chronic medications, ranges from 44.4% to 64.0%, which is considerably higher than that in age-matched non-cancer controls and the general population (8, 9). However, limited epidemiological studies have been conducted to evaluate medication use patterns among survivors of childhood cancer, who may be even more susceptible to the premature onset of late effects of cancer treatment and non-cancer-related medical conditions. One study reported that central nervous system (CNS) agents, hormone replacement therapy, and anti-infectives were more frequently prescribed to survivors of childhood cancer than to non-cancer controls (10). Similarly, compared with their non-cancer siblings, adolescent and adult survivors of childhood cancer were found to have a higher utilization of psychoactive prescription medications for managing pain and psychological symptoms (11, 12).

Unfortunately, although pharmacological intervention may confer therapeutic benefits to patients with multiple chronic conditions, an increased use of chronic medications increases the odds of having polypharmacy. It is well established in the literature that polypharmacy is associated with unwanted consequences such as an increased risk of drug–drug interactions, adverse drug events, and medication non-adherence in cancer patients (8, 13, 14). Mitigating these negative outcomes requires identifying subgroups of patients who are at risk of polypharmacy so their medication regimen can be improved. Certain late effects can occur early within a few years after completing cancer treatment (15), raising the question of whether the use of chronic medications is more prevalent among adolescent and young adult survivors of cancer than in the general population. Furthermore, little is reported about the prevalence and predictors of polypharmacy in survivors of childhood cancer.

The primary objectives of this study were to describe the pattern of chronic prescription medication use and estimate the prevalence of polypharmacy in survivors of childhood cancer. The secondary objective was to identify the clinical and treatment factors associated with medication burden and polypharmacy.

METHODS

Study Design and Setting

This was a retrospective cross-sectional study conducted between January 2019 and December 2019 at the Long-term Follow-up (LTFU) Clinic of the Prince of Wales Hospital in Hong Kong, a regional tertiary care public hospital that serves as one of the major hubs for providing LTFU care for survivors of childhood cancer.

Data on the treatment history and prescription medication use of the eligible survivors were extracted from the internal data repository and electronic patient record system of the study institution, known as the Clinical Data Analysis and Reporting System (CDARS) and Clinical Management System (CMS), respectively, and were retrospectively reviewed. The CDARS database includes patient-specific data, such as the clinical diagnosis, prescribed and dispensed medications, and information on hospitalization and discharge, that are entered by trained clinicians and healthcare professionals. Both the CDARS and CMS databases have been proven to be reliable sources of health administrative data for conducting epidemiological research in Hong Kong (16, 17). This study was approved by the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee (Ref. No. 2018.427).

Study Population and Setting

Survivors were eligible if they were diagnosed with cancer before the age of 18 years between January 1, 2000, and December 31, 2015. We included survivors who were diagnosed with cancer before the age of 18 years, as patients of this age range are typically treated under pediatric specialty care in the Hong Kong

healthcare system. Patients who were diagnosed prior to January 2000 were also excluded as the electronic CMS was in full operation only after 2000.

Eligible survivors were also (1) at least 3 years post-cancer diagnosis; (2) considered "end-of-treatment (EOT)," which is defined as more than 30 days from the last documented date of chemotherapy, immunotherapy, targeted therapy, radiation, or surgery; and (3) receiving active LTFU care at the study site between December 1, 2015, and September 30, 2018. This definition of survivorship was adopted to examine drug utilization in survivors from the early post-therapy phase to the long-term survivorship phase. This definition was also adopted in a similar study [Smitherman et al. (10)]. We excluded patients who (1) did not receive cancer treatment at the study institution; (2) developed secondary malignancies; or (3) were deceased at the time of data collection.

Study Outcomes

The primary outcomes were medication burden (the number of chronic prescription medications) and polypharmacy. Chronic medications were defined as medications prescribed to be taken daily for ≥30 days or used on an "as needed" basis for ≥6 months cumulatively within the past 12-month period. Each dispensed medication was counted as one medication item, regardless of the number of active ingredients. Medications were categorized according to the Anatomical Therapeutic Chemical Classification from WHO Collaborating Centre for Drug Statistics Methodology (18). Medications without active ingredients were excluded (**Supplement 1**).

For this study, we adopted the most popular and well-accepted definition of polypharmacy for the overall cohort, i.e., the use of ≥5 concurrent chronic medications (19).

Predictors

Based on a literature review (9–11), several predictive factors for chronic medication use were identified *a priori*. Clinical predictors included age at diagnosis, primary cancer diagnosis, chronic conditions, and years since EOT. Treatment predictors included chemotherapy, radiotherapy, surgery, and hematopoietic stem-cell transplantation (HSCT).

Data Analysis

Descriptive statistics were used to summarize the distribution of relevant outcome variables, predictors, and covariates according to reasonable groupings that are consistent with those in previous reports on childhood cancer (10, 11). Multivariable log-binomial models (generalized linear models with Poisson error and log-link function) were used to identify clinical and treatment factors associated with polypharmacy adjusted for sex and current age. Relative risk (RR) estimates and 95% confidence intervals (CIs) were reported. A similar multivariable log-binomial model was also used to identify clinical and treatment factors associated with specific therapeutic classes of chronic medications. Only the therapeutic classes used by more than 10% of the cohort were included in the analyses.

Finally, a Cox proportional hazards regression analysis was conducted to calculate hazard ratios (HRs) with 95% CIs for polypharmacy across years since diagnosis. HRs are presented for the variables identified in the previous multivariable log-binomial models for predicting polypharmacy, adjusting for sex. The Schoenfeld residuals were calculated for each variable to ensure that it independently satisfied the assumptions of the Cox model.

A series of sensitivity analyses were conducted. (1) Emerging evidence suggests an alternative definition of polypharmacy in the pediatric population, i.e., the use of ≥2 concurrent chronic medications (20). For a sensitivity analysis, we defined polypharmacy by this alternative definition in survivors aged less than 18 years. (2) Different international childhood cancer cohorts adopt varying diagnosis ages for childhood cancer, which include 0 to 15 years [British Childhood Cancer Survivor Study (21)], 0 to 18 years [Late Effects of Childhood Cancer task force of the Dutch Childhood Oncology Group (22) and Canadian PETALE (23)], and 0 to 21 years [United States Childhood Cancer Survivor Study (24) and Swiss Childhood Cancer Survivor Study (25)]. For a sensitivity analysis, we reported the rate of polypharmacy for survivors who were diagnosed at 0 to 15 years ("pediatric" cancer) and >15 years to 18 years ("adolescent" cancer). (3) The rate of polypharmacy was also reported separately for "early survivors" vs. "long-term" survivors (defined as ≤ 5 years post-diagnosis and > 5 years post-diagnosis, respectively). Association between age at diagnosis and time since diagnosis, adopting the above definitions in (2) and (3), was evaluated using a similar multivariable log-binomial model for sensitivity analysis. A P value of less than.05 was considered statistically significant, and all tests were two-sided. All analyses were conducted in SAS (SAS 9.4, SAS Institute, Cary, NC).

RESULTS

Study Population Characteristics

A total of 1213 patients were screened for eligibility (**Figure 1**). The final study population included 625 survivors of childhood cancer (mean current age = 17.9 years, standard deviation [SD] = 7.2 years) whose time since EOT was 9.2 [5.2] years (**Table 1**). The mean age at cancer diagnosis was 7.6 (5.34) years. The single largest group of survivors was diagnosed with leukemia (n = 275, 44.0%), followed by lymphoma (n = 70, 11.2%) and central nervous system (CNS) tumor (n = 66, 10.6%). The major treatment modalities were chemotherapy (n = 557, 89.1%) and surgery (n = 265, 42.4%). Among survivors who had undergone radiation therapy (n = 148, 23.7%), almost half had received cranial radiation (n = 73, 49.3%), followed by chest (n = 21, 14.2%) and abdominal (n = 19, 12.8%) radiation. Approximately one-tenth of the survivors (n = 68, 10.9%) suffered from at least one chronic health condition at the time of evaluation.

Pattern of Chronic Medication Use and Polypharmacy

Approximately one-third of the survivors (n = 219, 35%) were prescribed at least one chronic medication (**Table 2**). Among

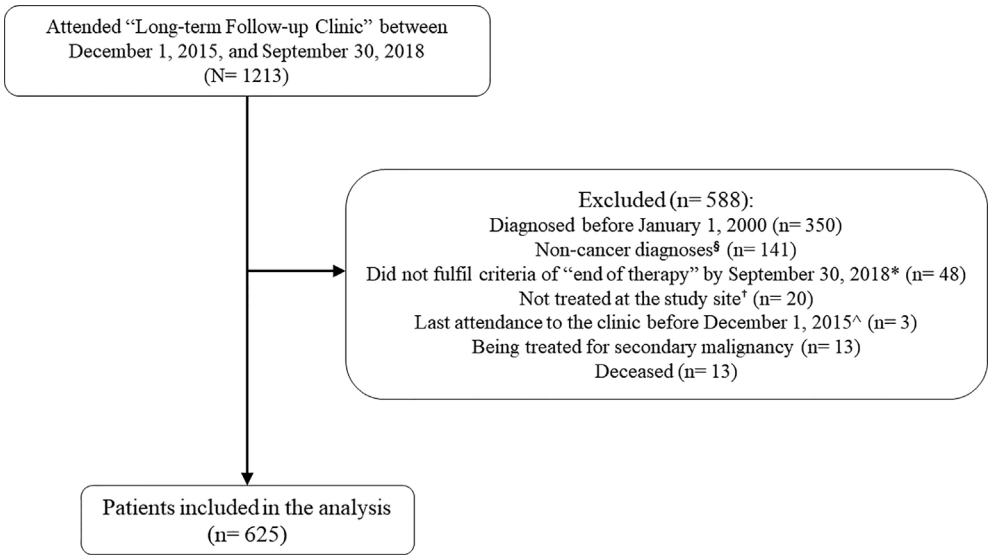

FIGURE 1 | Subject screening. § The "Long-term Follow-Up Clinic" of the study site (Prince of Wales Hospital) provides follow-up care for survivors of pediatric cancers and hematology diseases. Patients who were diagnosed with non-cancer conditions (e.g. aplastic anemia, thalassemia, etc.) were excluded. *"End-of-treatment" is defined as more than 30 days from the last documented date of chemotherapy, immunotherapy, targeted therapy, radiation, or surgery + Survivors not treated at the study site was excluded as we were not able to retrieve their treatment history. ^Survivors with stable health status are typically scheduled for a follow-up consultation once every 1 to 2.5 years. Survivors whose last attendance to the clinic was before December 2015 were likely lost to follow up.

these survivors, the median number of medications was 2 (interquartile range: 1–3). The most commonly prescribed medications belonged to the therapeutic classes of systemic antihistamines (n = 58, 26.5%), sex hormone replacement therapy (n = 42, 19.2%), thyroid therapy (n = 35, 16.0%), and systemic antimicrobials (n = 33, 15.1%). **Supplement 2** summarizes the therapeutic classes and specific medications prescribed to more than 5% of the cohort prescribed at least one chronic medication.

Collectively, the prevalence of polypharmacy (≥5 concurrent chronic medications) was 5.3% (n = 33) in the overall study cohort and 15.1% among survivors with chronic medication use. The rates of polypharmacy in clinically relevant subgroups of survivors are presented in **Supplement 3**. Polypharmacy was observed in 4.5%–5.9% of survivors aged less than 18 years, 4.9% of survivors aged 19–30 years, and 11.7% of survivors aged more than 30 years. In terms of cancer diagnosis, the rates of polypharmacy were 4.4%, 4.2%, and 13.6% in survivors of hematological malignancies, solid tumors, and CNS tumors, respectively (**Figure 2**). The rates of polypharmacy for "early survivors" (≤ 5 years post-diagnosis) and long-term survivors (>5 years post-diagnosis) were 7.0% and 4.8%, respectively (**Supplement 3**). Polypharmacy was also observed in a minority of survivors who had received radiation to the CNS (16.4%) and other body sites (5.3%), as well as in survivors who had undergone HSCT (13.3%).

When we adopted the alternative definition of polypharmacy (≥2 concurrent chronic medications) in the pediatric population, the proportion of pediatric survivors taking chronic medications experiencing polypharmacy and the rate of polypharmacy in the overall study cohort were found to be 22.5% and 15.9% (n = 99), respectively.

Factors Associated With Polypharmacy

Survivors of CNS tumors were more likely to have polypharmacy than survivors of non-CNS solid tumors (RR = 4.10, 95% CI = 1.52–11.0) (**Table 3**). Previous exposures to cranial radiation (RR = 6.31, 95% CI = 2.75–14.49) and HSCT (RR = 3.53, 95% CI = 1.59–7.83) were also associated with higher odds of having polypharmacy. Survivors who developed chronic health conditions had 7 times higher odds of having polypharmacy than survivors who did not have chronic conditions (RR = 7.35, 95% CI = 3.45–15.66).

For the multivariable Cox model (**Supplement 4**), the adjusted hazard ratios (HRs) for polypharmacy across years since cancer diagnosis showed significant association between polypharmacy and CNS tumor diagnosis (HR = 1.79, 95% CI = 1.25–5.07), chronic health conditions (HR = 3.39, 95% CI = 1.57–7.34), cranial radiation (HR = 3.34, 95% CI = 1.22 – 9.13) and bone marrow transplant (HR = 2.58, 95% CI = 1.19–5.62).

Factors Associated With Specific Therapeutic Classes of Medications

Subsequent analyses were performed to identify factors associated with the use of specific therapeutic classes of medications prescribed to more than 10% of the cohort (**Table 3**). The therapeutic classes included systemic antihistamines, sex hormone replacement therapy, thyroid therapy, and systemic antimicrobials. Older age was associated with the use of sex hormone replacement therapy (RR = 1.11,

TABLE 1 | Characteristics of study population (n = 625).

Characteristics	N (%)
Sex	
Male	358 (57.3)
Female	267 (42.7)
Current Age, years	
Mean (± SD)	17.9 (± 7.24)
≥3 to ≤ 6	22 (3.5)
>6 to ≤ 12	134 (21.4)
>12 to ≤ 18	146 (23.4)
>18 to ≤ 30	306 (48.9)
>30	17 (4.7)
Age at Diagnosis, years	
Mean (± SD)	7.6 (± 5.35)
*Pediatric cancer:**	
0 to ≤ 6	300 (48.0)
>6 to ≤ 12	155 (24.8)
>12 to < 15	97 (15.5)
*Adolescent cancer:**	
≥ 15 to ≤ 18	73 (11.7)
Time since Cancer Diagnosis, years	
Mean (± SD)	10.4 (± 5.2)
*≤5 years post-diagnosis:**	
≤ 3	37 (5.9)
> 3 to 5	105 (16.8)
*>5 years post-diagnosis:**	
>5 to 10	174 (27.8)
>10 to 15	148 (23.7)
>15	161 (25.8)
Time since End of Treatment, years	
Mean (± SD)	9.2 (± 5.18)
≤2	55 (8.8)
≤2 to 5	115 (18.4)
>5 to ≤ 10	175 (28.0)
>10 to ≤ 15	159 (25.4)
>15	121 (19.4)
Primary Cancer Diagnosis	
Hematological	
Leukemia	275 (44.0)
Lymphoma	70 (11.2)
CNS tumors	66 (10.6)
Other solid tumors	
Malignant bone tumors	48 (7.7)
Germ cell tumors	40 (6.4)
Neuroblastoma and other peripheral nervous cell tumors	33 (5.3)
Soft tissue carcinoma	32 (5.1)
Renal carcinoma	25 (4.0)
Other malignant epithelial neoplasms and malignant melanomas	14 (2.2)
Hepatic tumors	13 (2.1)
Retinoblastoma	5 (0.8)
Other and unspecified malignant neoplasms	4 (0.6)
Treatment	
Chemotherapy	557 (89.1)
Alkylating agents	483 (86.7)
Mustard gas derivatives	402 (72.2)
Heavy metals	158 (28.4)
Antitumor Antibiotics	476 (85.5)
Anthracyclines	430 (77.2)
Plant Alkaloids	380 (68.2)
Vinca Alkaloids	378 (67.9)
Antimetabolites	376 (67.5)
Folic acid antagonist	342 (61.4)
Pyrimidine antagonist	325 (58.4)
Purine antagonist	262 (47.0)
Topoisomerase inhibitors	238 (42.7)
Surgery	265 (42.4)
Radiotherapy	148 (23.7)
Cranial	73 (49.3)
Other body sites	75 (12.0)
Chest	21 (14.2)
Abdominal	19 (12.8)
Pelvic	16 (10.8)
Others	21 (3.4)
Total body	19 (12.8)
Bone marrow transplant	75 (12.0)
Other cancer therapies[§]	43 (7.7)
Chronic health conditions	68 (10.9)
Endocrine^	62 (9.9)
Metabolism	9 (1.4)
Neurological	5 (0.8)
Psychiatry	

(Continued)

TABLE 1 | Continued

*Subgroups and definitions adopted for sensitivity analyses.
[§]Includes immunotherapy, targeted therapy, hormonal therapy and other types of treatment.
^Includes hypopituitarism, growth hormone deficiency, gonadal dysfunction, thyroid dysfunction.

TABLE 2 | Pattern of prescription chronic medication use.

Overall cohort	N = 625
Use of prescription chronic medication	**n (%)**
Patients with ≥1 chronic medication	219 (35.0)
Patients with no chronic medication	406 (65.0)
Subgroup with prescribed chronic medication	n = 219
No. of concurrent chronic medications per patient	
Median (interquartile range) [range]	2 (1–3) [1–22]
1 to 2	143 (65.3)
3 to 4	43 (19.6)
5 to 6	20 (9.1)
7 to 8	7 (3.2)
>9	6 (2.7)
Therapeutic classification*	
Systemic antihistamines	58 (26.5)
Sex hormones replacement therapy	42 (19.2)
Thyroid therapy	35 (16.0)
Systemic antimicrobials	33 (15.1)
Drugs for obstructive airway diseases	21 (9.6)
Drugs for acid-related gastric disorders	17 (7.8)
Pituitary and hypothalamic hormones and analog	16 (7.3)

*Includes the therapeutic classes prescribed to more than 5% of the cohort prescribed with chronic medications (n = 219).

95% CI = 1.06–1.17) and thyroid therapy (RR = 1.07, 95% CI = 1.01–1.12), whereas younger age was associated with the use of systemic antihistamines (RR = 0.94, 95% CI = 0.91–0.98) and antimicrobials (RR = 0.87, 95% CI = 0.82–0.92).

Compared with survivors of non-CNS solid tumors, survivors of CNS tumors were more likely to be prescribed sex hormone replacement therapy (RR = 9.98, 95% CI = 3.74–26.66) and thyroid therapy (RR = 23.83, 95% CI = 7.45–76.20) (**Table 4**). More than one-fifth of CNS tumor survivors were prescribed sex hormone (22.7%) and thyroid (28.8%) therapies, as compared with less than 5% of survivors of other cancers (**Figure 2**). In contrast, survivors of hematological malignancies were more likely than survivors of solid tumors to be prescribed systemic

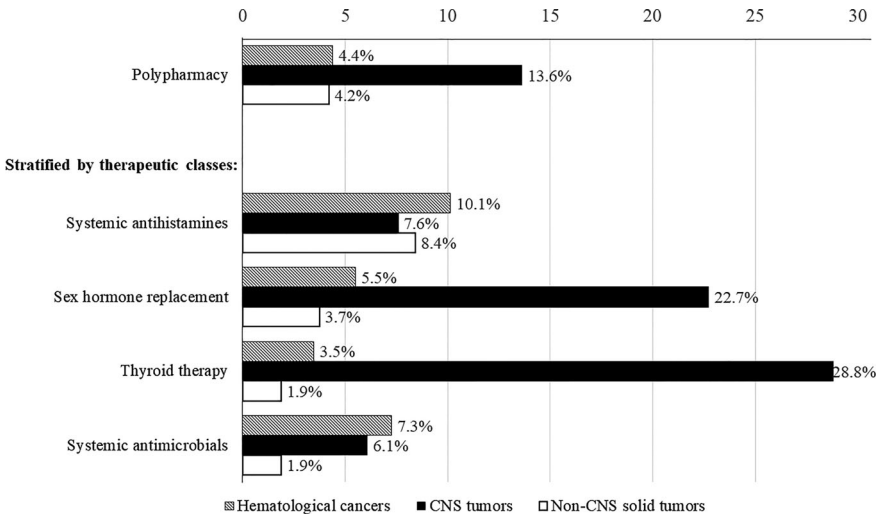

FIGURE 2 | Rates of polypharmacy and medication use stratified by cancer diagnoses. Includes only therapeutic classes used by more than 10% of survivors prescribed with chronic medications.

antimicrobials (RR = 6.72, 95% CI = 2.20–20.53) (**Table 4**). Having at least one chronic condition was associated with higher odds of receiving sex hormone replacement therapy (RR = 24.43, 95% CI = 11.6–51.45) and thyroid therapy (RR = 36.96, 95% CI = 16.06–85.02).

Sensitivity Analyses

A sensitivity analysis showed that when adopting the alternative definition of polypharmacy (≥2 concurrent chronic medications) in pediatric survivors, the predictors of polypharmacy were similar to those identified in the primary analysis (**Supplement 5**). However, associations became weaker, as reflected by the decrease in the magnitude of the risk ratio estimates (**Supplement 5**). Additional sensitivity analysis also did not show significant association between age at diagnosis (pediatric vs. adolescent) and time since diagnosis (≤ 5 years post-diagnosis *vs.* > 5 years post-diagnosis) with polypharmacy (**Supplement 6**).

DISCUSSION

Currently, limited drug utilization data on childhood cancer survivors are available in the literature. This study examined the prevalence and predictors of polypharmacy in a cohort of relatively young survivors of childhood cancer. We found that 35% of these young survivors were prescribed at least one chronic medication with polypharmacy detected in a minority of survivors. Our findings are consistent with the well-established evidence that certain subgroups of survivors are at a higher risk of developing multiple cancer treatment-related chronic health conditions that require pharmacological interventions (3, 7, 15, 26). Higher medication burden was found in HSCT recipients, who are known to suffer from a myriad of late effects due to intensive myeloablative conditioning treatment and total body irradiation. Sex hormones and thyroid medications were commonly prescribed to treat secondary endocrinopathy in survivors of CNS tumors and survivors who had undergone cranial radiation. These procedures typically involve higher radiotherapy doses of >30Gy and have a known association with endocrinopathy. Importantly, older age was associated with an increased use of hormone medications, which indicates that cancer survivors develop treatment-related chronic medical morbidities as they age. Survivors of hematological malignancies and CNS tumors were more likely to be persistently prescribed antimicrobials. This may reflect the subset of post-BMT patients with chronic graft-versus-host-disease (GVHD) and a small subgroup of patients who underwent splenectomy.

Our findings are comparable to those of a similar study by Smitherman et al. (10), who evaluated prescription drug use in survivors of childhood cancer in the United States using a commercial insurance claims database. This similarity most likely stems from the comparable contemporary cancer treatments adopted by the United States and Hong Kong. Both studies identified higher utilization of gonadal, pituitary, and thyroid replacement hormones and antimicrobials (antibacterials, antifungals, and antivirals) in survivors. However, it is worth noting that minor differences exist in drug use patterns between the two studies. First, antihistamines were the most commonly prescribed medications in our cohort but not in Smitherman et al.'s study cohort. The high utilization of prescribed systemic antihistamines in younger survivors in our study may be due to several reasons. First, the prevalence of allergies in the local pediatric population is often higher than western countries due to environmental factors. Secondly, systemic antihistamines are often prescribed to local patients

TABLE 3 | Factors associated with polypharmacy.

	Risk Ratio	95% CI		*P*
Demographic factors				
Sex				
Male	0.60	0.30	1.22	0.16
Female	*Ref*			
Current age at study	1.01	0.96	1.01	0.69
Clinical factors*				
Age at diagnosis	1.05	0.96	1.15	0.22
Years since EOT	0.93	0.85	1.02	0.15
Primary diagnosis				
Hematological	1.04	0.44	2.45	0.91
CNS	4.10	1.52	11.02	**0.0051**
Non-CNS solid tumor	*Ref*			
Chronic health conditions (any)				
Yes	7.35	3.45	15.66	**<.0001**
No	*Ref*			
Treatment factors*				
Chemotherapy				
Yes	0.65	0.24	1.77	0.40
No	*Ref*			
Radiation				
Cranial radiation	6.31	2.75	14.49	**<.0001**
Body only (Chest, abdomen, pelvis)	1.60	0.51	4.91	0.41
None	*Ref*			
Surgery				
Yes	1.13	0.56	2.30	0.72
No	*Ref*			
Bone Marrow Transplant				
Yes	3.53	1.59	7.83	**0.0019**
No	*Ref*			

CNS, central nervous system; EOT, end of treatment; Ref, reference group.
**Models are adjusted for demographic factors: sex and age at study.*
Bold: Statistical significance P < 0.05.

during their routine follow-up visits for improved affordability and convenience. In most western countries, these medications are often available as over-the-counter and do not require a prescription for purchase. Second, contrary to Smitherman et al.'s study, we did not identify a high rate of antihypertensive use in our cohort. Cardiovascular morbidities are more prevalent in survivors of lymphoma who are treated with high-dose anthracyclines and thoracic radiation (4, 27, 28). The higher proportion of lymphoma survivors in Smitherman et al.'s study cohort (26.4%) than in our study cohort (11.2%) may explain this difference. Third, Smitherman et al.'s study and reports from the Childhood Cancer Survivors Study (11, 12) in the United States found that survivors were more likely to use opioids and psychoactive medications, which were not common classes of prescription drugs found in our study cohort. We speculate that this difference in drug use pattern may be attributable to the lower utilization of psychotropic drugs and opioids in Asian populations (29, 30). Findings from our study may reflect the late chronic health problems experienced by subgroups of cancer survivors treated with contemporary regimens, and should be validated through a multi-centered study in Hong Kong, as well as collaboration with other international groups (31, 32). For example, the linkage of community-dispensed prescriptions data to cancer registration data in the United Kingdom can greatly enhance our understanding of the patient pathway and drug utilization of cancer patients (33, 34). As cancer treatment strategies evolve, up-to-date assessments of morbidities and medication use patterns are necessary to provide optimal risk-based survivorship care for survivors of childhood cancer.

The rate of polypharmacy (≥5 concurrent chronic medications) in our cohort of young survivors was low. However, it is worth noting that this estimate is higher than the reported rates of polypharmacy in the general population aged less than 40 years. When applying the same definition of polypharmacy, the rate of polypharmacy in individuals aged 20–39 years was 0.3% in one Dutch study (35) and 3.1% in one study from the United States (36). An Italian study reported that polypharmacy was detected in 2.98% and 3.29% of female and male subjects aged 15–64 years, respectively (37). In the general population of Japan, the rate of polypharmacy was 4.9% in those aged 20–34 years (38). All of these reported estimates from the literature seem to be lower than the polypharmacy rate of 5.3% observed in our study cohort. Importantly, we found that the rate of polypharmacy in survivors aged more than 30 years was considerably higher (11.7%). We acknowledge that this finding must be interpreted with caution due to the absence of an appropriate control group. However, it is reasonable to speculate that given the higher reported prevalence of chronic health conditions in childhood cancer survivors of the North American, European and Asian populations (5–7, 15, 22, 39),

TABLE 4 | Factors associated with specific therapeutic classes of medications.

	Systemic antihistamines				Sex hormone replacement				Thyroid therapy				Systemic antimicrobials			
	RR	**95% CI**		***P***	**RR**	**95% CI**		***P***	**RR**	**95% CI**		***P***	**RR**	**95% CI**		***P***
Demographic factors																
Sex																
Male	0.96	0.55	1.66	0.88	0.81	0.43	1.54	0.53	1.30	0.64	2.64	0.46	1.23	0.58	2.60	0.57
Female	*Ref*				*Ref*											
Current age at study	0.94	0.91	0.98	**0.0061**	1.11	1.06	1.17	**<.0001**	1.07	1.01	1.12	**0.0072**	0.87	0.82	0.92	**<.0001**
Clinical factors*																
Age at diagnosis	1.05	0.98	1.13	0.14	0.94	0.87	1.03	0.21	1.02	0.93	1.11	0.64	1.50	1.28	1.76	**<.0001**
Years since EOT	0.92	0.86	1.00	0.053	1.04	0.96	1.13	0.31	0.97	0.89	1.06	0.62	0.50	0.39	0.64	**<.0001**
Primary diagnosis																
Hematological	1.38	0.75	2.55	0.29	1.44	0.61	3.43	0.39	1.77	0.56	5.61	0.32	6.72	2.20	20.53	**0.0008**
CNS	0.94	0.33	2.68	0.91	9.98	3.74	26.66	**<.0001**	23.83	7.45	76.20	**<.0001**	4.50	1.04	19.34	**0.043**
Non-CNS solid tumor	*Ref*				*Ref*				*Ref*				*Ref*			
Chronic health conditions (any)																
Yes	1.54	0.68	3.44	0.29	24.43	11.60	51.45	**<.0001**	36.96	16.06	85.02	**<.0001**	0.72	0.16	3.18	0.66
No	*Ref*				*Ref*				*Ref*				*Ref*			
Treatment factors*																
Chemotherapy																
Yes	2.11	0.72	6.12	0.16	0.90	0.30	2.71	0.85	3.56	0.47	26.72	0.21	§			
No					*Ref*				*Ref*				*Ref*			
Radiation																
Cranial radiation	0.67	0.25	1.75	0.41	14.15	6.20	32.29	**<.0001**	13.81	5.73	33.27	**<.0001**	1.23	0.40	3.77	0.70
Body only (Chest, abdomen, pelvis)	0.61	0.21	1.80	0.37	6.96	2.85	17.01	**<.0001**	6.75	2.57	17.70	**0.0001**	1.28	0.35	4.59	0.69
None	*Ref*				*Ref*				*Ref*				*Ref*			
Surgery																
Yes	0.87	0.50	1.52	0.61	1.21	0.63	2.30	0.56	1.60	0.80	3.19	0.18	2.47	1.11	5.49	**0.026**
No	*Ref*				*Ref*				*Ref*				*Ref*			
Bone Marrow Transplant																
Yes	1.15	0.50	2.65	0.75	10.22	5.12	20.38	**<.0001**	3.93	1.84	8.36	**0.0004**	3.97	1.61	9.80	**0.0027**
No	*Ref*				*Ref*				*Ref*				*Ref*			

RR, relative risk; 95% CI, 95% confidence interval; Ref, Reference group.

**Models are adjusted for demographic factors: sex and age at study.*

§Association analysis was not conducted for variable "chemotherapy" as the sample size for non-chemotherapy receiving survivors treated with systemic antimicrobials was too low, hence yielding unstable parameter estimates.

Bold: Statistical significance P < 0.05.

their medication burden may be considerably higher than that of the general population. An additional point to note is that these are relatively young survivors and they are at risk of developing more chronic morbidities with longer follow up. Future work should compare the pattern of prescription drug use between survivors and age-matched controls, and determine whether the difference in drug utilization might be attributable to cancer treatment-associated medical problems in survivors.

We adopted a more conservative definition of polypharmacy (≥5 concurrent chronic medications) in the primary analysis. In our sensitivity analysis, the prevalence estimate of polypharmacy was even higher at 22.5% when the alternative definition of polypharmacy (≥2 concurrent medications) was adopted for the pediatric subgroup. We speculate that conducting this sensitivity analysis is well justified, as one recent scoping review reported that more than 80% of the studies defined polypharmacy as ≥2 concurrent medications or therapeutic classes in pediatric patients with chronic illnesses (20). Notably, 15% of our pediatric survivors were found to have been prescribed two or more concurrent chronic medications. This estimate is comparable to the rate of polypharmacy previously reported in children with autism spectrum disorder, psychiatric conditions, and epilepsy (40–42). The definition of polypharmacy for the pediatric population should have a lower threshold number of medications than that for the adult population, as children are expected to have a lower disease burden than adults. However, the use of multiple therapeutic classes of medications is likely warranted in "complex chronic conditions" such as childhood cancer (43). Unfortunately, the term "polypharmacy" often carries a negative connotation in the literature. More efforts are needed to streamline medication utilization and polypharmacy research in pediatric patients with chronic diseases. The development of clinical guidelines should also take into consideration both the benefits (efficacy and synergistic effects) and harms (cost, adverse effects, pill burden, and non-adherence) of combining medications when defining pediatric polypharmacy in survivors of childhood cancer.

The long-term use of multiple medications is associated with the risk of adverse drug events and increase in healthcare costs in survivors of cancer (8, 13, 14). This is especially concerning in settings where the survivor may be prescribed other acute medications in addition to the regular chronic medications. For example, variability in the absorption and metabolism of oral levothyroxine can occur when it is administered with proton pump inhibitors, antacids, and certain fluoroquinolones (44). In addition to sex hormones and thyroid replacement therapies, late

effects in HSCT recipients are typically treated with other chronic drugs such as steroid immunosuppressants, anxiolytics, antihypertensive drugs, and statins. One review examining post-HSCT sexual health highlighted that drug–drug interactions, pill burden, and combined adverse-effect profiles of medications may exacerbate sexual dysfunction in HSCT recipients (45). We propose that future work should evaluate health and humanistic outcomes associated with medication burden in high-risk groups of survivors. These studies will allow a better evaluation of potential drug-drug interactions and predictors of polypharmacy in this vulnerable population.

We inferred from our study findings that a closer monitoring of adverse health outcomes and drug interactions is warranted in survivors of CNS tumors and survivors who have undergone HSCT or cranial radiation. In terms of interventions, many studies on the cancer population have reported the benefits of having a pharmacist or interdisciplinary team to review medication use in patients with high medication burden (46–49). Such initiatives have successfully led to the reduction in potentially inappropriate or redundant medications, preventing drug interactions and promoting better medication-related behaviors in patients (50–52). Pharmacists as drug experts can perform routine medication reconciliation at every follow-up visit with the survivor and enquire about the use of over-the-counter drugs and CAM agents. This is especially relevant in the clinical setting of Hong Kong and China, where oncologists struggle with a heavy patient load. Furthermore, complementary/alternative medication (CAM) is becoming increasingly prevalent among children with cancer (53–56). Identifying clinical consequences of herb–drug interactions is an under-addressed problem in the field of cancer survivorship because few clinical trials have evaluated the concurrent use of CAM and Western medications in cancer survivors. There is a risk that CAM-related adverse events may not be detected or may be confounded with symptoms of late effects or adverse effects related to Western medications. Thus, pharmacists play an important role in counseling survivors on the appropriate use of medications and maintaining active communication between the oncologists and survivors regarding modifications to the medication list.

Despite having a relatively large, well-characterized sample with reliable sources of medication unitization data, the findings of this study should be considered in the context of several limitations. First, the CMS and CDARS databases of the Hospital Authority in Hong Kong vary in the quality of recorded health information (17). For example, late effects are dependent on the doses of radiation, but dosimetry data were not included in our study because of the inconsistency of available radiation records documented in our clinical databases. The calculated prevalence of chronic health conditions in our study cohort (10.9%) may not be a true reflection of the actual prevalence because survivors who presented with mild clinical presentation of late effects (e.g., Grade 1 conditions on the Common Terminology Criteria for Adverse Events Scale) may not have their conditions coded in the electronic health records. Further verification with the consultation notes is needed to validate disease coding in the CDARS. Second, based on geographical locations, there are seven clusters of public medical institutions in Hong Kong. The catchment area of the current study site included only the New Territories East Cluster. However, given the small cohort of healthcare professionals who specialize in pediatric oncology in Hong Kong, there is no reason to speculate that the treatment, healthcare, and drug utilization outcomes in our study cohort were any different from those in other clusters. Third, although we adjusted for demographic variables, medication use may not be directly attributable to the cancer treatment exposures, as there may be other confounding factors that were not captured in this study. For example, the relatively high rate of antihistamine use may be due to pre-existing allergic conditions that are not related to the cancer or the treatment exposures. Lastly, our estimation of medication burden did not include medication prescribed by private institutions, over-the-counter medications, and oral forms of CAM. Therefore, the true prevalence of polypharmacy may be even higher than the rate detected in this study.

CONCLUSION

Our findings suggest that although polypharmacy was observed in a minority of the included survivors in this study, chronic medication use was common occurrence and has the potential to contribute to future medical burden. Consistent with the literature, we found that treatment-related chronic morbidities develop gradually in survivors as they age and may compound the occurrence of other age-related chronic health conditions. Our study identified a higher medication burden was more prevalent in survivors of CNS tumors and survivors who had undergone cranial radiation or HSCT. One potential clinical implication is that such individuals should be monitored more closely for drug–drug interactions and adverse health outcomes that may result from multiple chronic medications, particularly during hospitalization in an acute care setting. Given that this is a single-centered study with a modest sample size, our findings should be validated in larger cohorts of childhood cancer survivors. Future studies should also focus on identifying factors affecting prescribing behaviors and drug utilization patterns in this specific population. It is anticipated that such real-word data may help us understand healthcare resource utilization and the potential impact of newer treatments in survivors of childhood cancer.

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 The Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee.

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

CE, YMC, HL, and YTC were responsible for the conceptualization and data analysis of the work. JW, AC, FP, and CL contributed in the study design and methodology. All authors contributed to the article and approved the submitted version.

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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.2021.642544/full#supplementary-material

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Neurocognitive and Behavioral Outcomes of Chinese Survivors of Childhood Lymphoblastic Leukemia

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Background: Increasing attention has been dedicated to investigate modifiable risk factors of late effects in survivors of childhood cancer. This study aims to evaluate neurocognitive and behavioral functioning in a relatively young cohort of survivors of childhood acute lymphoblastic leukemia (ALL) in Hong Kong, and to identify clinical and socio-environmental factors associated with these outcomes.

Methods: This analysis included 152 survivors of childhood ALL who were ≥5 years post-diagnosis (52% male, mean [SD] age 23.5[7.2] years at evaluation, 17.2[7.6] years post-diagnosis). Survivors completed performance-based neurocognitive tests, and reported their emotional and behavioral symptoms using the Child/Adult Behavior Checklist. Socio-environmental variables (living space, fatigue, physical activity, family functioning, and academic stress) were self-reported using validated questionnaires. Clinical variables and chronic health conditions were extracted from medical charts. Multivariable linear modeling was conducted to test identify factors associated with neurocognitive/behavioral outcomes, adjusting for current age, sex, age at diagnosis and cranial radiation. An exploratory mediation analysis was performed to examine the mediating effects of risk factors on neurocognitive and behavioral outcomes.

Results: As compared to population norms, a minority of survivors developed mild-moderate impairment in motor processing speed (36.2%), memory (9.2%) and attention measures (4.0%-10.5%). Survivors also reported attention problems (12.5%), sluggish cognitive tempo (23.7%) and internalizing (depressive, anxiety and somatic symptoms) problems (17.1%). A minority of survivors developed mild-moderate treatment-related chronic conditions (n=37, 24.3%). As compared to survivors without chronic conditions, survivors with chronic conditions had more executive dysfunction (B=5.09, standard error [SE]=2.05; P=0.014) and reported more attention problems (B=5.73, SE=1.43; P<0.0001). Fatigue and poor family functioning was associated with multiple measures of behavior problems (all P<0.001). A lower level of physical activity was correlated with

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more self-reported symptoms of inattention (B= -1.12, SE=0.38, *P*=0.004) and sluggish cognitive tempo (B=-1.22, SE=0.41, *P*=0.003). Exploratory analysis showed that chronic health conditions were associated with behavioral measures through fatigue as the mediator.

Conclusion: The majority of young Chinese survivors of ALL had normal cognitive and behavioral function. Regular monitoring of behavioral function should be performed on survivors who develop treatment-related chronic conditions. Health behavior and socio-environment factors may be potentially modifiable risk factors associated with health outcomes in survivors.

Keywords: cognitive, function, behavior, childhood cancer, childhood acute lymphoblastic leukemia (ALL), survivorship

INTRODUCTION

Improved treatment strategies for childhood acute lymphoblastic leukemia (ALL) have yielded survival rates higher than 90% (1). However, survivorship may be complicated by a myriad of treatment-related adverse effects (2, 3). Most current survivors of childhood ALL receive contemporary treatments that eliminate the need for cranial radiation therapy (CRT). Still, long-term survivors of childhood ALL who have been treated with contemporary chemotherapy protocols exhibit mild to moderate neurocognitive impairment (4, 5). The rates of neurocognitive deficits affecting executive function, processing speed and memory are threefold higher among survivors of childhood ALL than the general population (4, 6–8). Survivors also demonstrate behavioral and psychological problems (9–11), as well as worse academic performance (12, 13).

The association of central nervous system (CNS)-directed therapies, such as high-dose methotrexate and intrathecal chemotherapy, with worse neurocognitive outcomes in survivors of childhood ALL is well established (4, 6–8, 14–16). Leukoencephalopathy, sepsis and other acute toxicities that occur during active treatment are predictive of structural changes in the brain and subsequent deficits in functional outcomes (9, 17–19). After treatment, aging survivors of childhood cancer develop chronic health conditions, such as cardiovascular, pulmonary and metabolic disorders, at higher rates than those observed in age-matched non-cancer siblings (20, 21). In addition to their associations with early mortality, emerging studies of survivors have shown that these chronic health problems are related to cognitive impairment and psychosocial difficulties (15, 22–25).

To date, the majority of cognitive studies have involved Western populations. However, a recent systematic review identified 13 cognitive studies in survivors of childhood cancer in Asian countries, and found that 10.0%–42.8% of survivors demonstrated mild-to-moderate impairments in intelligence (i.e., overall IQ) (26). Evidence obtained in a Western population cannot be extrapolated to Asian survivors because of genetic differences in responses to drug therapies and susceptibilities to developing treatment-related chronic toxicities (27). Ethnic and sociocultural factors may lead to differential effects of treatments on cognitive processes in Asian and Western survivors (28, 29). Cultural values and family relationships may also shape psychosocial development (30). However, few studies have systematically evaluated the multifactorial aspects of cognitive and psychosocial outcomes in Asian survivors of childhood cancer.

Notably, most research has focused on either disease- or treatment-related factors as predictors of cognitive dysfunction. Few studies have examined the mediating effects of socio-environmental factors on the functional outcomes of survivors (31). This is especially relevant in the Asian context, in which great emphasis has been placed on ameliorating the adverse health effects of an urban environment, such as sleep disturbances, a sedentary lifestyle and academic stress, on children and adolescents (26, 32). Particularly, poor environmental factors and health behaviors may influence poorer neurocognitive and behavioral functions, especially in survivors who are already at risk of adverse health outcomes due to the cancer and related treatment.

In Hong Kong, approximately 50 pediatric patients are diagnosed with leukemia each year (33). The survival rate of patients with childhood ALL in Hong Kong is comparable to those in other developed countries, and more than 90% of patients survive more than 5 years after diagnosis (34). Currently, no studies have systematically characterized the functional outcomes in this population. The objectives of this study were to evaluate the prevalence of neurocognitive and behavioral deficits, and to identify clinical and socio-environmental factors associated with these outcomes in a cohort of young Chinese survivors of childhood ALL. We also included an exploratory objective to examine the mediating effects of socio-environmental factors on neurocognitive and behavioral outcomes in this population.

METHODS

Study Design

This was a prospective, cross-sectional study conducted at the Long-term Follow-up (LTFU) Clinic of the Prince of Wales Hospital in Hong Kong. This regional tertiary care public hospital serves as a major hub providing LTFU care to survivors of childhood cancer. This study was approved by the

Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee. Written informed consent and assent were obtained from all adult and pediatric participants, respectively.

Study Population

Eligible participants were recruited through consecutive sampling. Between June 2019 and June 2020, investigators obtained the list of patients who were due for follow-up consultation at the LTFU clinic, which typically occurred once a week. Patients were then screened for eligibility using the in-house electronic patient record system (Clinical Management System [CMS]). All eligible patients who subsequently attended the LTFU clinic were invited to participate in the study.

Survivors were eligible for the study if they (1) were at least 12 years old during the time of recruitment, (2) had been diagnosed with ALL before the age of 18 years, and (3) had survived for at least 5 years since diagnosis or had completed treatment at least 2 years previously. We excluded survivors who (1) had relapsed, (2) developed secondary malignancies, (3) had any genetic disorder or pre-existing condition associated with cognitive impairment (e.g., Down syndrome), (4) were pregnant or lactating or (5) had a history of traumatic brain injury.

Of the 192 survivors who were screened for eligibility, 175 fulfilled the inclusion criteria and were invited to participate in the study (**Supplement 2**). Subsequently, 158 survivors completed the assessments. After excluding 6 of those survivors because of missing diagnosis or treatment protocol information, the data of 152 survivors were analyzed (response rate: 86.9%). The study cohort (**Table 1**) comprised 32 pediatric survivors (mean age = 14.0, SD = 2.2 years) and 120 adult survivors (mean age = 26.0, SD = 5.9 years). On average, they were 17.2 (SD = 7.6) years post-cancer diagnosis, and 15.3 (SD = 11.2) years had elapsed since treatment (**Table 1**).

Previous Treatment Exposures

The treatment characteristics of the study cohort is presented in **Table 1**. All survivors in Hong Kong were treated with childhood ALL protocols (34) that were similar to the strategies adopted by international pediatric oncology organizations. Based on the clinical presentations of leukemia at diagnosis and evaluation of minimal residual disease, the patients were stratified into standard-risk, intermediate-risk, or high-risk protocols. Protocols were divided into four major components: remission induction block, consolidation block, maintenance block, and CNS-directed treatment. A variety of cytotoxic drugs were administered, which typically included intravenous (IV) high-dose methotrexate (HDMTX, defined as a single-dose of more than 1 g/m^2 of methotrexate) with leucovorin rescue, intrathecal chemotherapy injections (either methotrexate, or a combination of methotrexate, hydrocortisone, and cytarabine), oral dexamethasone pulses, anthracyclines, L-asparaginase, cytarabine and cyclophosphamide. Children who were stratified into the intermediate-risk/high-risk protocols received longer duration of treatment with higher intensity of

TABLE 1 | Clinical and Treatment Characteristics of Study Cohort (n=152).

Characteristics	No. (%)	Mean (SD)
Demographics and Clinical		
Sex		
Male	79 (52.0)	–
Female	73 (48.0)	–
Highest education (years)	–	13.2 (3.3)
Age at diagnosis (years)	–	6.3 (4.3)
Age at evaluation (years)	–	23.5 (7.2)
≤ 18	32 (21.0)	–
>18 – ≤30	93 (61.2)	–
>30	27 (17.8)	–
Time since diagnosis (years)	–	17.2 (7.6)
5 – ≤10	27 (17.8)	–
>10 – ≤15	35 (23.0)	–
>15 – ≤20	44 (28.9)	–
>20	46 (30.3)	–
Time since completion of treatment (years)	–	15.3 (11.2)
2 – ≤10	39 (25.6)	–
>10 – ≤15	35 (23.0)	–
>15 – ≤20	43 (28.3)	–
>20	35 (23.0)	–
Risk group		
Standard risk	61 (40.1)	–
Intermediate risk	67 (44.0)	–
High risk	22 (14.1)	–
Missing	2 (1.3)	–
Treatment modality		
Cranial radiation	32 (21.0)	–
Chemotherapy-only protocol	120 (79.0)	–
HSCT	4 (2.6)	–
Chemotherapy		
IV daunorubicin/doxorubicin* (mg/m^2)	–	194.4 (53.0)
IV high-dose methotrexate* (g/m^2)	–	14.2 (6.3)
8g/m^2	56 (36.9)	–
20 g/m^2	96 (63.1)	–
Intrathecal chemotherapy* (no. of injections)	–	17.6 (4.1)
Chronic health conditions^		
Any	37 (24.3)	–
Cardiopulmonary	13 (8.6)	–
Grade 1/2 (mild-moderate)	13 (8.6)	–
Grade 3/4 (severe-life threatening)	0	–
Endocrine	7 (4.6)	–
Grade 1/2 (mild-moderate)	7 (4.6)	–
Grade 3/4 (severe-life threatening)	0	–
Metabolic	7 (4.6)	–
Grade 1/2 (mild-moderate)	5 (3.3)	–
Grade 3/4 (severe-life threatening)	2 (1.3)	–
Neurology	10 (6.6)	–
Grade 1/2 (mild-moderate)	9 (5.9)	–
Grade 3/4 (severe-life threatening)	1 (0.6)	–
Psychiatry	9 (5.9)	–
Grade 1/2 (mild-moderate)	0	–
Grade 3/4 (severe-life threatening)	9 (5.9)	–
Vision & Hearing	3 (2.0)	–
Grade 1/2 (mild-moderate)	3 (2.0)	–
Grade 3/4 (severe-life threatening)	0	–

*HSCT, hematopoietic stem cell transplantation; IV, intravenous; SD, standard deviation. *Cumulative doses of selected chemotherapy drugs were extracted from medical charts, which were only available for 138 survivors. Cumulative doses for the remaining survivors (n=14) were estimated based on the chemotherapy protocol they received. Total HDMTX was also categorized as 8 g/m^2 (4 cycles of 2 g/m^2) of methotrexate versus 20 g/m^2 (4 cycles of 5 g/m^2).*

^Conditions were graded for severity according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE version 4.03).

HDMTX (4 cycles of 5g/m^2 HDMTX in intermediate-risk/high-risk protocols vs 4 cycles of 2g/m^2 HDMTX in low-risk protocols), intrathecal chemotherapy and dexamethasone, in addition to other chemotherapeutic agents. A minority of children in the intermediate-risk/high-risk arm, especially those who were treated before 1995, received prophylactic CRT.

Study Outcomes

Neurocognitive function was assessed using a standardized performance-based neurocognitive battery, which included: (1) *measures of attention* (Continuous Performance Test-III [CPT-III] variables: detectability, omissions, variability, hit reaction time block change and hit reaction time inter-stimulus interval) (35), (2) *memory* (Modified Taylor Complex Figure) (36), (3) *processing speed* (Trail Making Test Part A [TMT-A] and Grooved Pegboard) (37) and (4) *executive function* (Trail Making Test Part B [TMT-B] and CPT-III variables: commissions and preservations) (37). Detailed descriptions of the study tools and sources of reference norms data are reported in Supplement 1.

Behavioral functioning was evaluated using the Child Behavior Checklist (CBCL) and Adult Behavior Checklist (ABCL) for pediatric survivors (12 – 18 years of age) and adult survivor (≥ 18 years of age), respectively (38). The syndrome scales of the CBCL and ABCL were set as the primary behavioral outcomes of interest in this study. The standardized scales included attention problems, thought problems, internalizing problems (consisting anxiety/depression, somatic complaints and withdrawn behavior), externalizing problems (consisting aggressive behavior, intrusive behavior and rule-breaking behavior), obsessive-compulsive problems and sluggish cognitive tempo. Traditional Chinese-language versions of the ABCL and CBCL are available, and age- and gender-standardized local norms have been established for the Hong Kong context (39). The descriptions of ABCL/CBCL domains are presented in **Supplement 1**.

All neurocognitive and behavioral measures were transformed into age-adjusted *T*-scores (mean = 50; standard deviation [SD] = 10) using references provided by the test manuals or the published literature (**Supplement 1**). All *T*-scores were scaled such that a higher score was indicative of worse cognitive functioning or more severe problems. To estimate the prevalence of impairments within the study sample, impairment was defined as a score worse than 1.5 standard deviations of the age-adjusted *T*-score, a definition that has been adopted by multiple studies involving childhood cancer survivors in the literature (6, 10, 15, 18).

Clinical and Treatment Variables

Demographic and cancer-related information was abstracted from the Clinical Management System (CMS), an electronic health data repository of the public healthcare system in Hong Kong. This database is considered a reliable data source for epidemiological research in Hong Kong (40). The CMS includes cancer-related variables (diagnoses, age at diagnosis, risk stratification) and treatment-related variables (chemotherapy drugs, cumulative doses, CRT).

Information about chronic health conditions was collected from the CMS and through patient/proxy interviews. The severity of the conditions was graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE version 4.03) (41), which defines the severity of the health conditions as asymptomatic/mild symptoms (grade 1), moderate symptoms requiring minimal interventions (grade 2), severe/disabling symptoms requiring extensive interventions (grade 3), and life-threatening conditions (grade 4). For this study, the health conditions of interest were limited to the cardiopulmonary, endocrine, metabolic, psychiatric, neurological and hearing/vision systems, as these have been associated with neurocognitive function in cancer survivors (15, 22, 23, 25, 42). Only chronic health conditions with a reported age of onset during or after the completion of cancer treatment were included.

Socio-Environmental Variables

Family functioning was assessed using the Chinese Family Assessment Instrument (CFAI) (43). The CFAI is a 33-item tool that measures the domains of mutuality, communication and cohesiveness, conflict and harmony, parental concern and parental control. It has been validated within the general population in Hong Kong, with satisfactory test–retest reliability (Cronbach's alpha = 0.96) (44). The item scores are summed to yield total scores ranging from 33 to 165, and a higher score represents poorer family functioning.

Physical activity was self-reported using the validated Chinese University of Hong Kong: Physical Activity Rating for Children and Youth (CUHK-PARCY) (45, 46), which uses an 10-point scale to evaluate the level, intensity and frequency of physical activity performed by children and adolescents. The scale ranges from 0 to 10, with a higher rating indicating a more physically active lifestyle.

The subjective fatigue level was assessed using the Pediatric Quality of Life Inventory Multidimensional Fatigue Scale (PedsQL MFS) (47, 48). This scale comprises 18 items that evaluate the subjective sleep-rest fatigue, general fatigue and cognitive fatigue. The PedsQL MFS has demonstrated good internal consistency reliability, content validity and construct validity in the Chinese pediatric and young adult population (49). Each item is scored on a 100-point reverse Likert scale. A lower score indicates more severe fatigue.

Academic stress in survivors who were still attending school was assessed using the Education Stress Scale for Adolescents (ESSA). The 16-item ESSA addresses five latent variables: pressure related to studies, academic workload, concern about grades, self-expectation and despondency (50). The ESSA has been culturally adapted and validated in Chinese adolescents. The scale showed good internal consistency (Cronbach's alpha = 0.81) and test–retest reliability (intraclass correlation coefficient = 0.78) in Chinese adolescents (50). The total score ranged from 16 to 80, with a higher score indicating greater academic stress.

Sample Size

The primary outcome of interest was a statistical difference in the mean cognitive scores between survivors and reference norms

(mean = 50, SD = 10). At the inception of this study, there were no studies that reported performance-based cognitive outcomes in Asian survivors of childhood ALL. Hence, sample size calculation was based Western survivors of childhood ALL who received similar treatment regimens and neurocognitive testing at follow-up (7). The mean differences in cognitive scores between survivors and norms ranged from 0.25 to 2.3 SDs. Conservatively, to detect a 0.25 SD difference in scores between survivors and norms with a significance level α=0.05, the required sample size was 126 survivors.

Statistical Analysis

The sample characteristics and outcomes measures are summarized using descriptive statistics. As CRT is a well-established predictor of brain outcomes in survivors of childhood cancer (7, 15, 22), the clinical characteristics and neurocognitive/behavioral outcomes are presented separately for survivors who did and did not undergo CRT (CRT and non-CRT groups, respectively). The Mann-Whitney U test and Chisquare test were used to compare differences in characteristics between CRT and non-CRT groups.

A one-sample *t* test was used to compare the survivors' performance with population norms (*T*-score = 50). Only measures on which the survivors differed from the normative samples after correcting for false discovery rate (51), and demonstrated an impairment rate of more than 5% were included in subsequent analyses.

Multivariable general linear modeling (GLM) was used to identify the factors associated with neurocognitive and behavioral outcomes. The basic model included the current age, sex, age at diagnosis and CRT status. Subsequently, other independent variables of interest were added to the basic model and analyzed separately to avoid multi-collinearity. The following risk factors were determined *a priori* based on a literature review (6, 7, 10, 13, 16, 18, 22, 23, 52–54): (1) chronic health conditions; (2) treatment factors, including risk stratification, the IV HDMTX dose (8 g/m^2 vs 20 g/m^2) and number of intrathecal chemotherapy injections; and (3) socio-environmental factors, including fatigue, living space (≤ 600 vs > 600 square feet [55 m^2]), family functioning, physical activity and academic stress (only for current students). Unstandardized point estimates (B) and standard errors (SE) were used to quantify the effect size of the associations. Correction was not conducted for multivariable analysis because the risk factors of interest were identified a priori.

To address the exploratory objective, a Spearman's correlation test was conducted to test the relationships between the socio-economic variables. Mediation analyses were performed to examine the mediating effects of socio-environmental factors on neurocognitive and behavioral outcomes. To ensure that the model was meaningful and to reduce redundancy, only variables that were significantly associated with outcomes in the multivariable models and those deemed to be conceptually relevant based on consensus from the investigators were included in the mediation analyses. Mediation pathways were tested using the PROCESS algorithm for SPSS (55). Separate mediation models were run for each outcome measure. Survivors' current age, sex, age at diagnosis and CRT status were included as covariates within mediation models. Unstandardized point estimates and bootstrapped 95% CIs (BCCI) for the total indirect effect and specific indirect pathways were estimated. Missing data were handled using listwise deletion. All analyses were conducted using SPSS version 26 (Chicago, SPSS Inc.). A *P* value <.05 was considered statistically significant, and all statistical tests were two-sided.

RESULTS

Comparison Between CRT Group Versus Non-CRT Group

A minority of survivors (n = 32, 21.1%) received CRT; the others were treated with chemotherapy-only protocols (**Supplement 3**). There were more male survivors in the CRT group than in the non-CRT group (78.1% vs 45.0%, *P* <.001). On average, survivors in the CRT group were older (30.8 [6.8] vs 21.5 [6.0] years, *P* <.0001) and had survived for a longer time since diagnosis (24.0 [7.9] vs 15.4 [6.4] years, *P* <.0001) than those in the non-CRT group. The CRT group also had significantly higher proportions of survivors who were diagnosed with an endocrine (12.5% vs 2.5%, *P* = 0.016), metabolic (12.5% vs 2.5%, *P* = 0.016) or neurological (18.8% vs 3.3%, *P* = 0.002) condition, compared with the non-CRT group.

Socio-Environmental Factors

The socio-environmental characteristics of the study cohort are presented in **Supplement 4**. The mean CUHK-PARCY score for physical activity was 6.0 (SD = 1.6, range 1 = 10), indicating that survivors generally participated in moderate physical activities for durations > 20 minutes once or twice per week. The survivors' self-reported fatigue score was 68.3 (SD = 14.8, range = 34.7 – 100). The mean family functioning score was 68.1 (SD = 21.4, range = 33 – 172). A slight majority (n = 90, 59.2%) of the survivors resided in less than 600 square feet of living space.

Neurocognitive and Behavioral Outcomes

Survivors performed more poorly than the reference norms on measures of motor processing speed, memory, executive function, and attention after correcting for false discovery rate (all *P* values <.05; **Figure 1**). The mean scores of all cognitive measures are presented in **Supplement 5**. A minority of survivors demonstrated impairments in memory (9.2%), motor processing speed (36.2%) and executive function on CPT Commissions (8.5%) (**Figure 1**). The rates of impairment on attention measures ranged from 4.0% to 10.5% (**Figure 1**).

Compared with the age- and sex-matched norms, the survivors reported significantly more issues with all measures of behavioral functioning (all *P* values <.001; **Figure 2**). The mean scores of all behavioral measures are presented in **Supplement 5**. On the syndrome scales, the proportions of survivors who reported symptoms of inattention and internalizing and externalizing problems ranged from 7.9% to 17.1%. Approximately a fifth of survivors reported thought

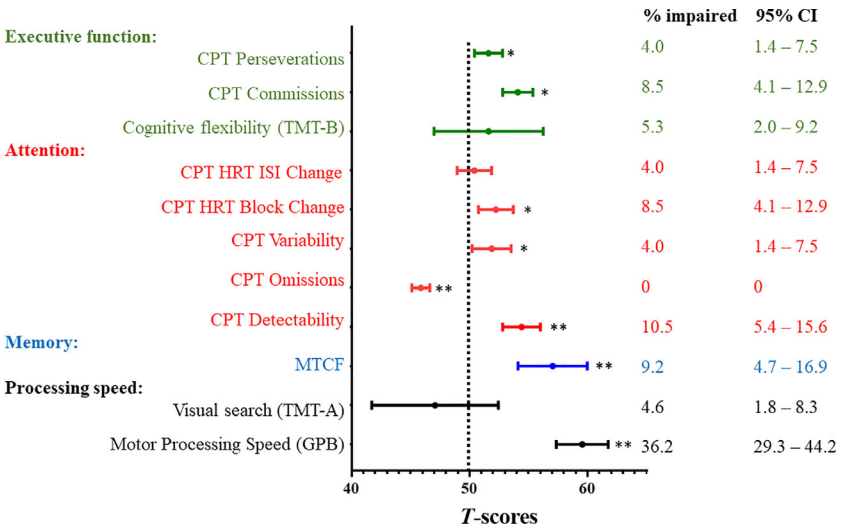

FIGURE 1 | Neurocognitive Outcomes and Prevalence Rates of Impairment. CI, confidence interval; CPT, Conners Continuous Performance Test-III; GPB, Grooved Pegboard; HRT, hit reaction time; ISI, inter-stimulus Intervals; MTCF, Modified Taylor Complex Figure; SD, standard deviation; TMT, Trail Making Test. All neurocognitive measures were transformed into age-adjusted *T*-scores (mean = 50; standard deviation [SD] = 10) using references provided by the test manuals or the published literature (**Supplement 1**). All *T*-scores were scaled such that a higher score was indicative of worse functioning. A one-sample *t* test was used to compare the survivors' performance with population norms (Dotted line; *T*-score = 50). * indicates statistical significance at $P \leqq 0.05$ after correcting for false discovery rate ** indicates statistical significance at $P \leqq 0.01$ after correcting for false discovery rate To estimate the prevalence of impairments within the study sample, impairment was defined as a score below the 1.5 standard deviation poorer than the age-adjusted *T*-scores of reference norms. Prevalence estimates are expressed as proportion (%) and 95% confidence intervals.

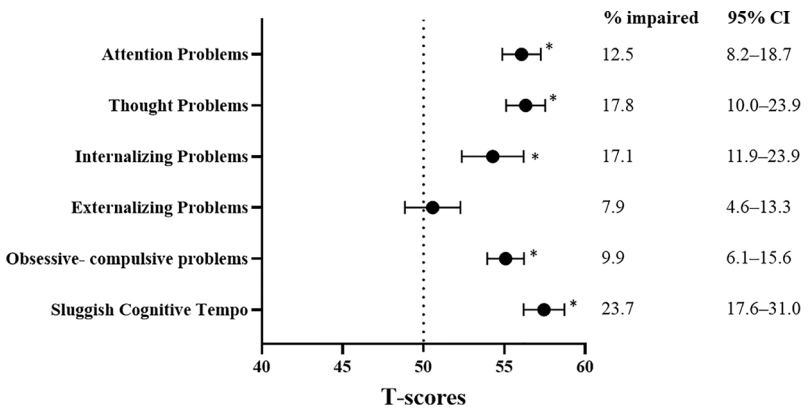

FIGURE 2 | Behavioral Outcomes and Prevalence Rates of Impairment. CI, confidence interval; SD, standard deviation. All behavioral measures were transformed into age-adjusted *T*-scores (mean = 50; standard deviation [SD] = 10) using references provided by the test manuals or the published literature (**Supplement 1**). All *T*-scores were scaled such that a higher score was indicative of more problems. A one-sample *t* test was used to compare the survivors' performance with population norms (Dotted line: *T*-score = 50). * indicates statistical significance at $P \leqq 0.01$ after correcting for false discovery rate. To estimate the prevalence of impairments within the study sample, impairment was defined as a score below the 1.5 standard deviation poorer than the age-adjusted *T*-scores of reference norms. Prevalence estimates are expressed as proportion (%) and 95% confidence intervals.

problems (17.8%), and nearly a quarter reported a sluggish cognitive tempo (23.7%).

Survivors in the CRT group demonstrated worse cognitive flexibility on TMT-B than those in the non-CRT group (mean [SD] 60.7 [28.2] vs 48.9 [20.9], $P = .050$) (**Supplement 6**). The CRT group reported more obsessive-compulsive problems ($P = 0.048$) and anxiety problems ($P = 0.015$) than the non-CRT group (**Supplement 6**). There were no between-group differences in the other neurocognitive and behavioral domain scores. Descriptively, the impairment rates for internalizing problems (31.3% vs 13.3%), externalizing problems (12.5% vs 6.7%) and obsessive-compulsive problems (18.8% vs 7.5%) were almost twice as high in the CRT group as in the non-CRT group.

Factors Associated With Neurocognitive Outcomes

After adjusting for sex, age at diagnosis, age at evaluation and CRT status, multivariable analysis showed that survivors who developed chronic health conditions had more executive dysfunction than those who did not have any chronic conditions (CPT commission: B = 5.09, SE = 2.05, *P* = .014) (**Table 2**). Interestingly, higher number of intrathecal chemotherapy injections was associated with a better performance on executive function (CPT commission: B = -0.024, SE = 0.09, *P* = .049, **Table 2**).

Survivors who resided in < 600 square feet of housing space had worse performances in the domains of memory (B = 6.50, SE = 2.88; *P* = .027) and motor processing speed (B = 4.75, SE = 2.21; *P* = .034) than survivors who resided in larger housing spaces. Worse family functioning was associated with more executive dysfunction (CPT commission: B = 0.07, SE = 0.04, *P* = .049). No associations of neurocognitive outcomes with other socio-environmental variables were observed.

Factors Associated With Behavioral Outcomes

Compared with survivors without chronic health conditions, those who developed chronic health conditions reported more symptoms of inattention (B = 5.75, SE = 1.43, *P* <.0001) and internalizing (B = 5.99, SE = 2.40, *P* = .014), externalizing problems (B = 4.62, SE = 2.16, *P* = .034), and sluggish cognitive tempo (B = 5.34, SE = 1.56, *P* = .001; **Table 3**). Survivors who were treated with a cumulative dose of 20 g/m^2 of HDMTX had more externalizing problems than survivors treated with 8 g/m^2 of HDMTX (B = 5.31, SE = 2.36, *P* = .027).

Regarding the living environment, poorer family functioning was strongly associated with all measures on the syndrome scales (**Table 3**). Survivors who resided in < 600 square feet of housing space were more likely to report internalizing problems (B = 4.06, SE = 1.99, *P* = .044) and a sluggish cognitive tempo (B = 2.89, SE = 1.31, *P* = .030) than survivors with larger housing spaces.

A higher level of physical inactivity was correlated with more self-reported symptoms of inattention (B = -1.12, SE = 0.38, *P* = .004) and a sluggish cognitive tempo (B = -1.22, SE = 0.41, *P* = .003; **Table 3**). Survivors who were more fatigued reported significantly worse outcomes on all measures of the syndrome scale (all *P* values <.0001). Among the subset of survivors who were current students, academic stress was associated with multiple domains of behavioral functioning (all *P* values <.05).

Exploratory Analysis

A positive correlation was also observed between physical inactivity and fatigue (r = 0.34, *P* <.0001), and strong inter-correlations were identified between fatigue, family functioning and academic stress (**Supplement 8**).

TABLE 2 | Factors Associated with Neurocognitive Outcomes.

Risk factors*	Motor processing speed (Grooved Pegboard)^			Memory (MTCF)^			Inattentiveness (CPT Detectability)^			Inattentiveness (CPT Commissions)^			Sustained attention (CPT HRT block change)^		
	B	SE	*P*	B	SE	*P*	B	SE	*P*	B	SE	*P*	B	SE	*P*
Treatment factors															
Risk group															
Intermediate/high risk	0.96	2.4	0.68	1.19	3.2	0.71	2.39	1.77	0.18	0.62	1.59	0.70	0.97	1.76	0.58
Standard risk (referent)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IV HDMTX [ɪ]															
20 g/m^2	2.14	3.37	0.53	6.42	4.59	0.17	0.39	2.31	0.86	1.98	2.54	0.44	2.83	2.22	0.21
8 g/m^2 (referent)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IT chemotherapy (no. of injections)	0.005	0.34	0.98	0.14	0.47	0.75	0.20	0.25	0.43	-0.024	0.09	**0.049**	-0.039	0.02	0.052
Chronic health conditions															
Any	4.94	2.75	0.074	7.87	3.98	0.051	2.94	2.01	0.14	5.09	2.05	**0.014**	-0.49	1.83	0.78
No (referent)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Socio-environmental factors															
Living space[※]															
≤ 600 square feet	4.75	2.21	**0.034**	6.50	2.88	**0.027**	1.96	1.68	0.24	1.15	1.75	0.51	1.41	1.53	0.36
>600 square feet (referent)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Family functioning^	0.06	0.05	0.23	-0.09	0.06	0.16	0.04	0.04	0.34	0.07	0.04	**0.049**	-0.02	0.03	0.53
Physical activity#	0.49	0.75	0.52	0.83	0.95	0.38	-0.69	0.53	0.19	-1.05	0.55	0.059	0.08	0.48	0.85
Fatigue#	-0.05	0.07	0.55	-0.14	0.09	0.14	-0.09	0.05	0.13	-0.11	0.05	0.070	0.06	0.05	0.20
Academic stress^,[§]	0.10	0.16	0.53	0.09	0.21	0.65	-0.07	0.09	0.48	-0.04	0.11	0.72	0.15	0.11	0.17

B, unstandardized coefficient; CPT, Conners Continuous Performance Test; HDMTX, high-dose methotrexate; HRT, hit reaction time; IT, intrathecal; IV, intravenous; MTCF, Modified Taylor Complex Figure; SE, standard error.

All statistical models were adjusted for sex, age at diagnosis, age at evaluation and cranial irradiation. Only measures on which the survivors differed from the normative samples after applying false discovery rate, and had an impairment rate of more than 5% (Figure 2**) were examined for associations with risk factors. Boldface indicates statistical significance at P≦0.05.*

^A higher value was indicative of worse functioning.

#A higher value was indicative of better functioning.

[ɪ]Total HDMTX categorized as 8 g/m^2 (4 cycles of 2 g/m^2) of methotrexate versus 20 g/m^2 (4 cycles of 5 g/m^2).

[§]Academic stress was evaluated in survivors who were still schooling.

TABLE 3 | Factors Associated with Behavioral Outcomes.

Risk factors*	Attention problems^			Thought problems^			Internalizing problems^			Externalizing problems^			Obsessive-compulsive problems^			Sluggish cognitive tempo^		
	B	SE	*P*	B	SE	*P*	B	SE	*P*	B	SE	*P*	B	SE	*P*	B	SE	*P*
Treatment factors																		
Risk group																		
Intermediate/ high risk	0.32	1.25	0.79	0.19	1.24	0.87	0.18	2.09	0.92	0.43	1.87	0.81	1.57	1.19	0.19	0.18	1.40	0.89
Standard risk (referent)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IV HDMTX [ɪ]																		
20 g/m^2	0.78	1.53	0.61	0.65	1.71	0.70	2.96	2.72	0.27	5.31	2.36	0.027	0.99	1.65	0.55	1.05	1.83	0.56
8 g/m^2 (referent)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IT chemotherapy (no. of injections)	0.002	0.01	0.89	0.005	0.01	0.73	-0.02	0.02	0.37	-0.04	0.01	**0.042**	-0.01	0.01	0.27	-0.015	0.01	0.35
Chronic health conditions																		
Any	5.73	1.43	**<0.0001**	3.05	1.53	**0.048**	5.99	2.40	**0.014**	4.62	2.16	**0.034**	1.12	1.43	0.43	5.34	1.56	**0.001**
No (referent)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Socio-environmental factors																		
Living space[※]																		
≤ 600 square feet	1.91	1.23	0.12	0.79	1.27	0.53	4.06	1.99	**0.044**	0.29	1.81	0.87	0.46	1.18	0.69	2.89	1.31	**0.030**
>600 square feet (referent)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Family functioning^	0.14	0.03	**<0.0001**	0.15	0.03	**<0.0001**	0.29	0.04	**<0.0001**	0.23	0.04	**<0.0001**	0.13	0.03	**<0.0001**	0.15	0.03	**<0.0001**
Physical activity#	-1.12	0.38	**0.004**	-0.87	0.39	**0.030**	-1.04	0.63	0.10	-0.71	0.57	0.21	-0.60	0.37	0.10	-1.22	0.41	**0.003**
Fatigue#	-0.30	0.03	**<0.0001**	-0.28	0.04	**<0.0001**	-0.53	0.05	**<0.0001**	-0.38	0.05	**<0.0001**	-0.24	0.03	**<0.0001**	-0.35	0.03	**<0.0001**
Academic stress^,[§]	0.21	0.08	**0.017**	0.16	0.09	0.08	0.49	0.13	**<0.0001**	0.38	0.11	**0.002**	0.19	0.08	**0.028**	0.19	0.09	**0.044**

B, unstandardized coefficient; HDMTX, high-dose methotrexate; IT, intrathecal; IV, intravenous; SE, standard error.

**All statistical models were adjusted for sex, age at diagnosis, age at evaluation and cranial radiation. Boldface indicates statistical significance at P≦0.05.*

*^A higher value was indicative of worse functioning. Refer to **Supplement 2** on detailed explanation of specific behavioral domains.*

#A higher value was indicative of better functioning.

[ɪ]Total HDMTX categorized as 8 g/m^2 (4 cycles of 2 g/m^2) of methotrexate versus 20 g/m^2 (4 cycles of 5 g/m^2).

[§]Academic stress was evaluated in survivors who were still schooling.

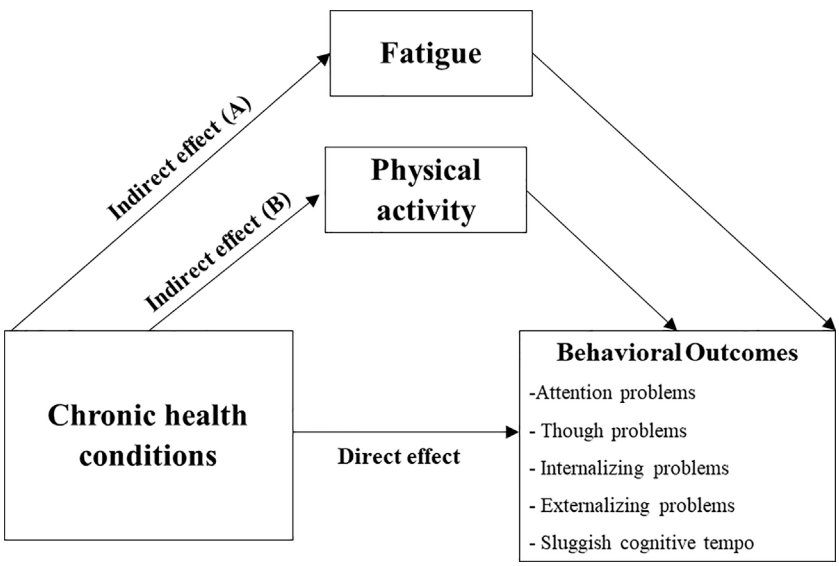

FIGURE 3 | Predicted Mediation Pathway (Exploratory Analysis). Predicted mediation pathway for association between chronic health conditions and behavioral outcomes, *via* fatigue **(A)** and physical activity **(B)**. Mediation analysis was run separately for each behavioral measure, controlling for sex, age at evaluation, age at diagnosis and cranial radiation.

Based on findings from the primary analyses and consensus from the investigators, we explored the mediating effect of fatigue on the relationship between chronic health conditions and self-reported behavioral outcomes, adjusting for current age, age at diagnosis and CRT status (**Figure 3A**). The analysis was then repeated, replacing fatigue with physical activity as the mediator (**Figure 3B**).

Chronic health conditions were significantly associated with self-reported attention problems indirectly through fatigue (ß = 3.06, $P = 0.007$; total indirect effect ß = 3.12, 95% BCCI 1.27 – 5.81). Similar results were obtained for other behavioral measures on the syndrome scales, except for thought problems (**Supplement 9**). Physical activity was not identified as a mediator between chronic health conditions and behavioral outcomes. Unstandardized point estimates and 95% BCCIs for the total indirect effect and specific indirect pathways are summarized in **Supplement 9**.

DISCUSSION

This is one of the first studies to evaluate the neurocognitive and behavioral outcomes of Chinese survivors of childhood ALL. A minority of survivors (4.0% to 36.2%) demonstrated moderate impairment on performance-based cognitive assessments across the domains of attention, executive function, motor processing speed and memory. Our results are consistent with those of studies in which ALL survivors were shown to exhibit deficits in these domains (4, 7, 26). Survivors reported more behavioral problems relative to the population norms, particularly in the domains of attention, a sluggish cognitive tempo and depressive symptoms.

Consistent with the literature (7, 15, 22), we found that the CRT group displayed significantly worse memory performances than the non-CRT group. The lack of differences in other neurocognitive and behavioral scores between the CRT and non-CRT survivors is probably due to the limited sample size in the former group. However, the rates of deficits in internalizing and emotional problems were descriptively higher in the CRT group. Specifically, a third of the survivors in the CRT group reported internalizing problems, such as depression, anxiety and withdrawal behaviors. Brain injuries induced by the cancer itself or by neurotoxic treatments, such as CRT, at an early stage in life can severely affect critical periods of brain development (7). Beyond the direct neurotoxic effect of CRT, the effects of aging and CRT-associated chronic health conditions may have contributed to the higher rates of functional impairment in the CRT group (15, 22). Hence, our results are consistent with well-established evidence indicating that survivors exposed to CRT face the highest burden of morbidity and require intensive cognitive and behavioral rehabilitation.

Fortunately, most contemporary international treatment protocols for ALL have eliminated CRT. Collective evidence from the literature on long-term survivors demonstrates the probable cognitive-sparing effect of chemotherapy-based protocols over CRT (4, 7, 8, 31, 56). However, survivors still suffer from apparent cognitive impairment due to the administration of intensive chemotherapy.

Although methotrexate is an integral component of modern ALL treatment regimen with known neurotoxic effects, we did not identify a significant and consistent dose-dependent relationship between HDMTX and long-term outcomes. Possibly, the cumulative dose is a less sensitive surrogate for drug exposure, as it may introduce errors of measurement and compromise the accuracy of the results (6). We also did not manage to account for the psychological effect of leucovorin rescue in this retrospective study. Interestingly, the use of IT chemotherapy was associated with better outcomes, probably because IT chemotherapy is a common component of contemporary treatment regimens and is typically associated with better functional outcomes than traditional CRT-based therapies in earlier eras. This probably explained why further post-hoc analysis showed that the association between IT chemotherapy and neurocognitive outcomes were no longer significant when the analysis was conducted only within the non-CRT group. Our future work will include the prospective collection of plasma HDMTX exposure and leucovorin rescue data as a more precise approach to identify the sources of long-term neurocognitive outcomes.

The association of specific treatment exposures at an early stage of life with health morbidities over time has been well established in survivors of childhood cancer, particularly in CRT survivors (20, 21). Our cohort of Chinese survivors was still relatively young. Still, nearly a fifth of the non-CRT survivors had developed clinical cardiopulmonary complications, psychiatric disorders and other health conditions. Notably, these chronic health conditions were associated with worse outcomes on multiple self-reported behavioral measures, even after accounting for CRT status. This finding is similar to those of emerging studies that identified the contributions of chronic health conditions resulting from childhood cancer therapies to emotional distress and neurocognitive impairment (22, 23, 25). These observations have important clinical implications. First, although the chronic health conditions in these survivors may be irreversible, the timely treatment of late effects may help to alleviate the psychological and behavioral symptoms. Hence, our findings emphasize the importance of systematic screening for chronic health conditions in survivors; to the best of our knowledge, this is not commonly practiced at most institutions in China (32). Second, the routine monitoring of psychological symptoms in young survivors who have developed chronic health conditions may facilitate the early identification of behavioral problems before they develop into clinical developmental problems and affect functional outcomes.

Our results offer valuable new insights regarding culturally relevant socio-environmental factors that influence behavioral functioning in Chinese survivors of ALL. We found that a smaller living space was associated with worse memory and motor processing speed performances on performance-based measures. Poorer family functioning correlated with more self-reported behavioral problems. General population studies have shown that environmental characteristics, such as noise

pollution, overcrowding and housing problems, can affect neurodevelopment in children (57–59). Although we did not have data on the household size and living space per person, this finding is still especially relevant to the context of Hong Kong, where the type, area and size of housing are surrogate markers of socio-economic status and access to quality healthcare (60, 61). Also notable is that the assessments of survivors in this study were conducted in 2019, during which Hong Kong was affected by citywide social unrest. Increases in family conflicts and emotional distress were reported during that period (62). Similarly, academic stress is widespread in most Asian societies, particularly in Chinese cultures (63, 64). From a service perspective, our findings highlight the importance of identifying these potentially modifiable risk factors and developing culturally relevant preventive interventions (e.g., providing social support to improve the family environment, mindfulness interventions to relieve academic stress) to improve the clinical and psychosocial outcomes of survivors.

Consistent with the literature (54, 65), in our study, fatigue was consistently associated with multiple measures of behavioral functioning. The survivors' self-reported fatigue score (mean = 68.3 points) corresponded to a mild-to-moderate level of fatigue, based on the published thresholds in individuals with major conditions (66, 67). Particularly, the results of our exploratory pathway analysis support the mediating effect of fatigue on the relationship between chronic health conditions and behavioral outcomes. Although physical activity was not a significant mediator of this relationship, it is strongly associated with both fatigue and measures of behavior functioning. Taken together, these preliminary findings complement the growing evidence in the literature that supports the beneficial effects of physical activity on mental health in cancer survivors (65, 68–70). From a physiological perspective, neurobehavioral changes are closely related to aging in the general population (71). Exercise may potentially delay the onset of premature frailty and chronic disease burdens and could lead to better psychosocial outcomes in survivors (72, 73). From a psychological perspective, certain types of sports promote teamwork and self-efficacy. The emotional and educational influences of physical activities may address specific externalizing problems in adolescents, such as rule-breaking behaviors, aggression and antisocial personality traits (74). For example, adventure-based and family-centered physical activity programs have been shown to reduce fatigue, depression and anxiety in survivors in both domestic and international studies (68, 75, 76).

Our study has a few limitations. This single-center study recruited survivors through non-probability sampling, which may have been subject to sampling bias. However, a post-hoc analysis did not identify major differences between participants and non-participants, except for a marginal older age at follow-up for the non-participant group (**Supplement 10**). The population of childhood ALL survivors in Hong Kong is small and we attempted to recruit every eligible survivor from consecutive sampling at the LTFU clinic; this approach would have established a reasonable sampling frame. While we acknowledge that the treatment protocols represented in our study cohort are heterogeneous and span across 1990s to 2000s, the treatment agents (except CRT) remain the backbone of modern therapies for childhood ALL. The treatment strategies across the eras are no different from protocols captured in epidemiological studies of other countries (22, 77–81). By including survivors who were treated in the earlier eras, our cohort is representative of the growing population of aging cancer survivors in the current health care system. We did not include a control group of subjects without cancer for comparison. However, the rates of impairment presented in this study were based on reference norms (Chinese norms for TMT-A, TMT-B and behavioral assessments), and our data do suggest that the survivors demonstrated worse behavioral outcomes relative to the general population. Although our sample size was calculated *a priori* to detect differences in cognitive outcomes between survivors and norms, it may not be sufficiently powered to detect associations with multiple risk factors. However, our findings are consistent with the robust literature that identifies CRT, fatigue and chronic health conditions as poor predictors of functioning. A minority of survivors in our sample were diagnosed before 2000, or prior to the implementation of computerized medical records, and therefore had incomplete medical and treatment records. For example, the total CRT doses and cumulative doses of most chemotherapy drugs could not be calculated because of incomplete documentation. Furthermore, a common source bias might explain the strong associations between self-reported behavioral outcomes and socio-environmental factors.

Future studies should include a more objective evaluation of variables, such as actigraphy studies physical activity and sleep, as well as culturally relevant measures of social attainment outcomes (e.g. housing tenure, employment history, personal income level etc.). Despite these limitations, this work serves as both a feasibility study and a model with which to facilitate larger-scale research that will validate our preliminary findings. To the best of our knowledge, this is one of the largest studies to examine the long-term neurocognitive and psychosocial outcomes of Chinese survivors of childhood ALL. A multicenter study that involves the prospective collection of outcome data and comparison with an age- and sex- matched control group may also better reflect the trajectories of functional outcomes in these Chinese survivors as they advance from early to long-term survivorship.

CONCLUSION

Our findings suggest that the majority of young survivors of ALL exhibit normal cognitive and behavioral function during the early phase of survivorship. However, subgroups of survivors who developed chronic health conditions or were exposed to adverse socio-environmental conditions were found to be at risk of developing poor functional outcomes. These individuals require closer pre-emptive screening to identify behavioral and cognitive problems before they develop into clinical developmental conditions that could impair the survivors' educational and occupational outcomes. We acknowledge that

it may not be possible to fully eliminate the neurotoxic effects of chemotherapy. Nevertheless, survivors may benefit from appropriate interventions to address modifiable risk factors, such as the provision of social support to families and interventions to encourage survivors to adopt a physically active lifestyle. Our findings should be validated in a larger-scale study that involves the prospective collection of outcome data, as this would better reflect the trajectories of neurocognitive and behavioral changes in survivors of childhood ALL in Hong Kong. We also expect that multinational and collaborative trials might facilitate comprehensive investigations of racial/ethnic-specific outcomes and the associated risk factors.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Conception or design of the work: All. Data acquisition: LP, PY, LY, YTC. Data analysis: LP, PY, YTC. Data interpretation: All. Drafting of report: LP, PY, YTC. Revising it critically for important intellectual content: All. All authors contributed to the article and approved the submitted version. Agreement to be accountable for all aspects of the work: All.

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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.2021.655669/full#supplementary-material

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Cardiac Surveillance for Early Detection of Late Subclinical Cardiac Dysfunction in Childhood Cancer Survivors After Anthracycline Therapy

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Background: In childhood cancer survivors (CCSs) anthracycline-related cardiotoxicity is an important cause of morbidity and late mortality, but the optimal modality of cardiac surveillance still remains to be defined. The aim of this study was to assess whether non-invasive echocardiography-based functional cardiac measures can detect early subclinical myocardial changes in long-term pediatric cancer survivors who received anthracycline therapy.

Methods: Twenty anthracycline-treated long-term CCSs and 20 age, sex, and body surface area matched healthy controls were enrolled in this study. Among cancer survivors, mean age at diagnosis was 6.5 ± 4.4 years, and the mean cumulative anthracycline dose was 234.5 ± 87.4 mg/m^2. All subjects underwent a comprehensive functional echocardiographic protocol study including two-dimensional echocardiography (2D Echo), tissue Doppler imaging (TDI), speckle tracking (STE) and three-dimensional echocardiography (3D Echo). Patients were studied at a mean follow-up time of 6.5 ± 2.8 years from the end of therapy.

Results: No significant differences in two-dimensional left ventricle ejection fraction (LVEF), diastolic parameters and speckle tracking (STE)-derived myocardial strain were observed between patients treated with anthracyclines and controls. Myocardial performance index was significantly prolonged (p = 0.005) and three-dimensional LVEF was significantly reduced (p = 0.002) in CCSs compared to controls, even though most values were within the normal range. There were no significant correlations between 2D, STE, and 3D echocardiographic parameters and age at diagnosis or duration of follow-up. No significant differences in echocardiographic parameters were found when stratifying cancer patients according to established risk factors for anthracycline cardiomyopathy.

Conclusions: This study found significantly reduced three-dimensional LVEF in CCSs compared with controls, despite no significant differences in two-dimensional LVEF and longitudinal strain values. These findings suggest that long-term CCSs who had received anthracycline therapy may be found to have subclinical features of myocardial dysfunction. However, further studies are needed to demonstrate the validity of new imaging techniques, including STE and 3D Echo, to identify patients at risk for cardiomyopathy in the long-term follow-up of CCSs.

Keywords: childhood cancer, anthracycline, late-onset cardiotoxicity, two-dimensional echocardiography, tissue Doppler imaging, speckle tracking echocardiography, three-dimensional echocardiography

INTRODUCTION

Advances in treatment strategies for childhood cancer have resulted in a significant improvement in survival (1, 2), but there is growing concern about the long-term side effects of therapy (3, 4). Among the most used chemotherapy drugs, anthracycline is a cornerstone in the treatment of several neoplastic diseases, including acute lymphoblastic leukemia, rhabdomyosarcomas, neuroblastoma, acute myeloid leukemia, Hodgkin lymphoma, osteosarcoma, and Ewing's sarcoma. However, the use of these drugs is strongly conditioned by their short and long term cardiac toxicity (4–6)

Left ventricle (LV) dysfunction and heart failure (HF) are the most concerning cardiovascular complications of cancer therapies and could increasingly become a main cause of morbidity and death of childhood cancer survivors s (CCSs) (7, 8).

The early detection of cardiotoxicity is often challenging, due to a long subclinical phase of LV dysfunction; however, an early diagnosis of cardiovascular disease in these patients would be crucial as it could allow a timely initiation of cardioprotective therapies that might delay myocardial remodeling and prevent LV cardiomyopathy.

Because of its widespread availability and safety, two-dimensional echocardiography (2D Echo) is standardly utilized in monitoring cancer patients, although it is not always adequate to detect an early, clinically asymptomatic stage of disease (9). Recently, new imaging approaches, including tissue Doppler imaging (TDI), speckle tracking echocardiography (STE), and three-dimensional echocardiography (3D Echo), have been utilized for early detection of asymptomatic LV cardiac dysfunction (10).

The aim of this study was to assess whether advanced non-invasive echocardiography-based methods can be helpful in detecting early subclinical myocardial dysfunction in childhood anthracycline-exposed cancer survivors.

MATERIALS AND METHODS

Study Population

This is an observational case–control study investigating 20 CCSs who received anthracycline therapy compared with 20 healthy subjects matched according to age, sex, and body surface area.

CCSs patients were followed at the Pediatric Oncology Unit of the Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy and were evaluated between October 2019 and February 2020. All patients received anthracycline therapy by a central line, with anthracycline infusion lasting 4 h.

Inclusion criteria for the study were: completion of anthracycline therapy for more than 3 years; cumulative anthracycline dose <360 mg/m^2; no symptoms of HF and normal global LV systolic function [defined as a LV ejection fraction [LVEF] ≥55%, and a LV shortening fraction (LVSF) ≥28%] at the last standard echocardiogram. Exclusion criteria included a history of congenital cardiac defects or any other cardiac disease; mediastinal/chest radiotherapy; bone marrow or staminal cells transplantation; signs and/or symptoms of acute and early cardiotoxicity. Healthy subjects were children referred to our Pediatric Cardiology Unit for complaints such as chest pain or palpitation, who were found to have normal cardiac examination, electrocardiogram, and standard transthoracic Doppler echocardiography.

Prior to the participation, the patients and their parents provided signed consent forms after being informed about the aim of the project as foreseen by the Italian Law on Privacy and the Safeguarding of the Sensitive Data (D.LGS n196, 2003). The study was carried out in accordance with the Helsinki declaration of human rights. Approval by the Ethics Committee was not necessary since echocardiography is recommended as the primary cardiomyopathy surveillance modality for assessment of left ventricular systolic function in follow-up of survivors treated with anthracyclines.

Clinical Characteristics

Demographic characteristics, blood pressure (BP), heart rate (HR), body mass index (BMI), and body surface area (BSA) were collected from all subjects. Type of malignancies, age at diagnosis, time since the last anthracycline dose, cumulative anthracycline dose, non-modifiable risk factors (female sex, age <5 years at diagnosis, concomitant administration of other chemotherapeutic agents, concomitant radiotherapy, cumulative anthracycline dose >250 mg/m^2, renal failure), and modifiable cardiovascular risk factors (hypertension, obesity, smoking, sedentary habit) were collected from CCSs patients.

LV Echocardiographic Study

All the subjects underwent a comprehensive functional echocardiographic protocol study including two-dimensional echocardiography (2D Echo), tissue Doppler imaging (TDI), speckle tracking (STE), and three-dimensional echocardiography (3D Echo).

All echocardiographic studies were performed by an experienced physician (a cardiologist expert in non-invasive imaging, together with a pediatric cardiologist), using a Philips EPIQ 7C® ultrasound system with a Philips X5-1 probe (Philips Medical System, Andover, Massachusetts, USA). Standard imaging planes were acquired according to current guidelines and recording three beat loops saved in DICOM format and stored in a workstation. These digital loops were interpreted by the same physicians, together, and both were blind to the subject's group.

First, we assessed LV morphology and function by M-mode and 2D echo. Standard echocardiographic parameters included LV end-diastolic (LVEDD) and end-systolic (LVESD) diameters, LV end-diastolic (LVEDV) and end-systolic (LVESV) volumes, interventricular septum (IVS) and posterior wall (PW) thickness. Measurements were indexed for BSA. As surrogates of LV systolic function, we determined the LVSF on M-mode imaging, LVEF by biplane Simpson's calculation, the mitral annular plane systolic excursion (MAPSE) and the myocardial performance index (MPI). MPI is an index that incorporates both systolic [isovolumic contraction time (IVCT) and LV ejection time (LVET)] and diastolic [isovolumic relaxation time (IVRT)] time intervals. It is, therefore, an expression of both global systolic and diastolic LV function and is calculated with the following formula: IVCT + IVRT/LVET. An MPI ≤0.40 is considered to be normal (11). LV dimension and function assessment was performed according to the recommendations of the American Society of Echocardiography (ASE) and the European Association of Cardiovascular Imaging (EACVI) (12, 13).

Diastolic function was assessed following the recommendations of the last guidelines (14). The following parameters were obtained by pulsed wave Doppler and TDI: 1) E/A wave ratio, *i.e.*, the ratio between the peak of early diastolic Doppler mitral blood flow velocity (E wave) and the peak of late diastolic Doppler mitral blood flow velocity (A wave); 2) septal and lateral E/E′ wave ratio, *i.e.*, the ratio between the E wave peak and the peak of the early diastolic myocardial velocity (E′ wave) on tissue Doppler imaging.

Then, STE was used to quantify myocardial deformation ("strain") as an index of myocardial function (15). Peak regional and global LV longitudinal strain was obtained from the apical two-, three- and four-chamber views. The best single cardiac cycle was selected for the analysis. After placing two mitral annular points and one apical point for each view, borders were traced semi-automatically and, if unsatisfactory, manually edited. The software automatically generated deformation curves and a "bull's eye" diagram as a topographic intuitive representation of the values obtained for each of the 17 myocardial segments (16). According to vendor-specific indications, strain values were considered normal if > 20%.

Lastly, we evaluated three-dimensional LV volumes and LVEF. 3D Echo is a novel echocardiographic technology that is based on the acquisition of "volumes" containing heart structures (the so-called 3D datasets). 3D measurements of volumes and LVEF have been proved to be more accurate and reproducible compared to standard 2D Echo and similar to those obtained with cardiac magnetic resonance (CMR) (17, 18). For each subject, three datasets were acquired and stored. The best single-beat volumetric dataset was selected for the analysis. Once identified the end-diastolic and the end-systolic frames, four mitral annular points and one apical point were placed as markers. Then LV endocardial borders were automatically traced by the software and manually adjusted when indicated.

Statistical Analysis

Continuous variables are reported as means and standard deviations, while categorical variables as numbers and percentages. The analysis of variance test was used to compare continuous variables, whereas nominal variables were compared by Fisher exact test. Correlation analysis was done by Pearson's test. Statistical significance was set at a p value <0.05.

RESULTS

The main demographic and clinical characteristics of the two groups are summarized in **Table 1**. As expected, no differences were observed for age, sex and anthropometric data.

Data about the neoplastic disease and treatment of CCSs patients are summarized in **Table 2**. As shown, age at cancer diagnosis was 6.50 ± 4.39 years; the time from the completion of chemotherapy was 6.50 ± 2.74 years, whereas the cumulative anthracycline dose was 234.50 ± 87.38 mg/m^2.

Acute lymphoblastic leukemia was the most common form of disease, followed by Hodgkin lymphoma, non-Hodgkin lymphoma and Ewing's sarcoma; only one patient was affected by neuroblastoma. At the time of the present study, all CCSs were asymptomatic and did not refer any physical limitation.

TWO-DIMENSIONAL ECHOCARDIOGRAPHY AND TISSUE DOPPLER IMAGING

Conventional echocardiographic parameters are shown in **Table 3**.

No significant differences in LV dimensions and volumes were found between the two groups. SF was slightly lower in CCS patients compared to controls, although values were still within

TABLE 1 | Clinical characteristics of CCSs and healthy subjects.

Clinical characteristics	CCSs	Healthy controls	p value
Age (years)	13.20 ± 2.78	12.45 ± 2,91	0.410
Male [n (%)]	11 (55)	13 (65)	0.531
Weight (kg)	50.90 ± 15.16	56.10 ± 18.66	0.457
Height (cm)	155.05 ± 15.58	156.70 ± 18.77	0.814
BMI	20.69 ± 3.56	20.47 ± 4.59	0.867
BSA (m²)	1.44 ± 0.26	1.49 ± 0.28	0.610

TABLE 2 | Specific characteristics of CCSs group.

CCS group	
Age at diagnosis (years ± SD)	6.50 ± 4.39
Years since last anthracycline dose (years ± SD)	6.50 ± 2.74
Cumulative anthracycline dose (mg/m² ± SD))	234.50 ± 87.38
Type of malignancies	**N (%)**
Acute lymphoblastic leukemia	8 (40)
Hodgkin lymphoma	3 (15)
Non-Hodgkin lymphoma	3 (15)
Ewing sarcoma	5 (25)
Neuroblastoma	1 (5)

the normal range (34.20 ± 4.29 *vs* 38.80 ± 7.26, respectively; p = 0.027). Furthermore, there was no significant difference in LVEF.

Global myocardial function evaluated through MPI resulted significantly lower in CCS patients than in controls (0.62 ± 0.14 *vs* 0.49 ± 0.03, respectively; p = 0.005). The difference was related to a significant increase in IVCT in patients (79.83 ± 16.50 *vs* 67.90 ± 9.48, respectively; p = 0.046), whereas there were no significant differences in LVET and IVRT between the two groups.

Diastolic parameters were normal in both groups, but the E/A ratio was significantly lower in CCS patients compared to controls (1.61 ± 0.42 *vs* 2.05 ± 0.69, respectively; p = 0.046). In CC patients, an inverse correlation was found between the E/A ratio and the cumulative anthracycline dose (r = −0.520; p = 0.023; **Figure 1**).

Speckle Tracking Imaging and 3D Echocardiography

Comparisons between CCSs and controls of STE parameters and 3D Echo parameters are shown in **Tables 4** and **5**, respectively.

Values of global strain on STE were normal in both groups, with no statistically significant differences. Furthermore, when stratifying for each segment, no differences were found in terms of regional longitudinal strain.

On 3D Echo imaging, LVEF was found significantly lower in CCSs compared with healthy controls (63.93 ± 3.87 *vs* 69.83 ± 5.75; p = 0.002), although values were within the normal range in all subjects. No significant differences in LV 3D volumes were found comparing patients and control group.

Finally, in CC patients there were no significant correlations between echocardiographic parameters (2D, STE, and 3D assessment) and age at cancer diagnosis or duration of follow-up. Furthermore, no significant differences were found in echocardiographic findings when patients were divided according to sex, age at diagnosis <5 years, cumulative anthracycline dose >250 mg/m² and concomitant radiotherapy (data not shown).

DISCUSSION

A growing number of CCSs have to face lifelong side effects of cancer therapies, some of which affecting the cardiovascular system (3, 4, 6). Indeed, these patients have significant long-term morbidity related to their cancer therapy and cardiovascular events are the leading non-malignant cause of death. Compared with the general population, CCSs are at a 15-fold increased risk of developing congestive HF (19) and at a seven-fold increased risk of premature death due to cardiac causes (20). A variety of cardiovascular complications are increasingly appearing among CCSs, including dilated

TABLE 3 | Conventional echocardiographic assessment.

Parameter (Mean ± SD)	CCS (20)	Healthy Controls (10)	p value
FS, %	34.20 ± 4.29	38.80 ± 7.26	0.027
Biplane Simpson's, EF %	64.90 ± 3.76	65.30 ± 4.42	0.810
EDD/BSA (mm/m²)	29.55 ± 4.03	29.90 ± 6.74	0.856
ESD/BSA (mm/m²)	19.65 ± 3.39	18.40 ± 3.92	0.374
IVSd/BSA (mm/m²)	5.20 ± 1.13	5.60 ± 1.42	0.448
PWd/BSA (mm/m²)	5.00 ± 1.15	5.50 ± 1.39	0.337
LVM/BSA (g/m²)	63.70 ± 12.15	74.20 ± 9.70	0.192
EDV (ml)	72.60 ± 19.80	81.70 ± 28.30	0.378
ESV (ml)	25.80 ± 7.80	29.00 ± 11.80	0.452
MAPSE (mm)	16.40 ± 2.60	17.70 ± 1.49	0.157
E (cm/s)	98.10 ± 15.24	105.40 ± 24.27	0.328
A (cm/s)	63.10 ± 13.78	53.70 ± 15.21	0.103
E/A ratio	1.61 ± 0.42	2.05 ± 0.69	0.046*
IVCT (ms)	79.83 ± 16.50	67.90 ± 9.48	0.046*
IVRT (ms)	94.00 ± 31.94	76.10 ± 11.31	0.101
ET (ms)	277.38 ± 28.68	290.40 ± 19.51	0.214
MPI	0.62 ± 0.14	0,49 ± 0,03	0.005*
E' L (cm/s)	18.47 ± 3.34	20.93 ± 4.55	0.110
E/E' L	5.76 ± 1.45	4.85 ± 0.63	0.069
E' M (cm/s)	12.42 ± 1.74	12.80 ± 1.60	0.573
E/E′ M	7.98 ± 1.43	7.00 ± 2.01	0.136

FS, fractional shortening; EF, ejection fraction; EDD, end-diastolic dimeter; ESD, end-systolic diameter; EDV, end-diastolic volume; ESV, end-systolic volume; E, early diastolic mitral inflow velocity; A, late diastolic mitral inflow velocity; IVRT, isovolumic relaxation time; IVCT, isovolumic contraction time; ET, ejection time; MPI, myocardial performance index; E', early diastolic mitral annulus velocity; E/E', ratio of early (E) mitral Doppler peak flow to early diastolic myocardial velocity (E').
**represent the value < 0.05 (that is the statistical significant value).*

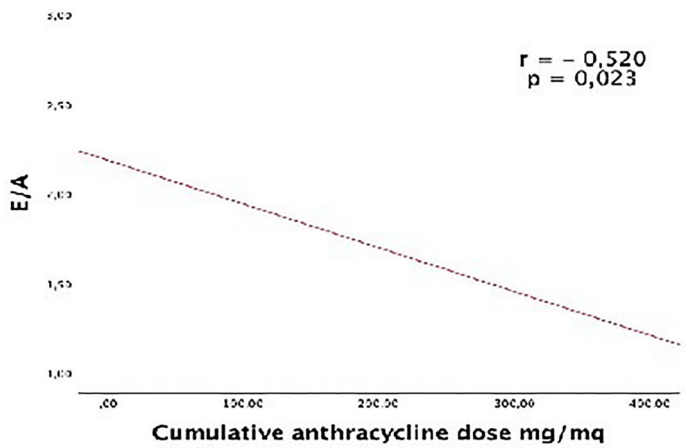

FIGURE 1 | Correlation between the E/A ratio and the cumulative anthrax cycline dose.

cardiomyopathy, myocardial infarction, valvular abnormalities, and pericarditis (21).

Among chemotherapeutics, anthracycline are widely used because of their high efficacy for treatment of childhood hematological malignancies and solid tumors, but are also those most commonly implicated in cardiac damage, resulting in an anthracycline-induced cardiomyopathy (22).

There is a strong dose-dependent relationship between anthracycline chemotherapy exposure and risk of congestive HF, with the incidence of cardiac dysfunction also increasing over time (4). Congestive HF and other adverse cardiovascular outcomes, indeed, may appear as late as 30 years after therapy (23). Of note, several studies have shown that cardiovascular complications may occur even after exposure to very low doses of the drug (23–25).

The cardiotoxicity of anthracycline may be acute, early or late (22). Acute cardiotoxicity occurs within the first week of anthracycline treatment; it is a transient depression of myocardial contractility and usually reversible when anthracycline are discontinued. Early onset cardiotoxicity occurs within the first year after the completion of anthracycline treatment; it results in dilated cardiomyopathy that can be a progressive disease. Late-onset cardiotoxicity occurs more than 1 year after the completion of anthracycline treatment and usually consists of dilated cardiomyopathy, which can show a progressive worsening over time.

Although characteristics and course of anthracycline-induced cardiotoxicity are still to be fully elucidated, the most commonly accepted pathophysiological mechanism is oxidative stress. According to this theory, the generation of reactive oxygen species and lipid peroxidation of the cell membrane can induce progressive cardiac remodeling as a late consequence of earlier myocyte injury, resulting in late cardiomyopathy (3).

Known risk factors for developing anthracycline-related cardiomyopathy include a cumulative dose higher than 250 mg/m^2, infusion regimen, age younger than 5 years, additional radiation therapy, other concomitant chemotherapies and female sex (26). Other conditions seem to increase cardiac susceptibility, like hypertension and obesity; furthermore there are emerging data suggesting that genetic susceptibility could also have a role in modifying the individual response to therapeutic exposures (27, 28).

The time point when late cardiotoxicity becomes clinically manifest varies substantially. Many affected patients may initially be asymptomatic, with clinical manifestations appearing several years later, with a subsequent continuous progressive decline in myocardial function (3, 4). If anthracycline-associated cardiac dysfunction is detected early and treated with appropriate medications, patients frequently have a good functional recovery. Conversely, if patients are identified late after the onset of cardiac dysfunction, HF is typically more difficult to treat (5, 26, 29). For this reason, cardiac dysfunction should be detected as earlier as possible. This could be achieved through a

TABLE 4 | Left ventricular strain characteristics measured by 2D speckle-tracking echocardiography among childhood cancer survivors and controls.

STE parameters (Mean ± SD)	CCS	Healthy Controls	P value
GLS 4CH %	22.17 ± 2.14	21.88 ± 1.85	0.718
GLS 3CH %	21.54 ± 2.61	21.12 ± 2.39	0.669
GLS 2CH %	22,58 ± 2,90	22,52 ± 3,00	0.958
Mean GLS	22,12 ± 1,86	21,99 ± 1,88	0.859

GLS, global longitudinal strain; 4Ch, apical-4 chambers view; 3Ch, apical-3 chambers view; 2Ch, apical-2 chambers view.

TABLE 5 | Left ventricular assessment measured by RT-3D echocardiography among childhood cancer survivors and controls.

RT-3D parameters (Mean ± SD)	CCS	Healthy Controls	P value
EDV (ml)	73.50 ± 25.99	84.07 ± 30.14	0.328
ESV (ml)	26.63 ± 9.88	25.76 ± 11.74	0.832
EF %	63.93 ± 3.87	69.83 ± 5.75	0.002*

EDV, end-diastolic volume; ESV, end-systolic volume; EF, ejection fraction.
**represent the value < 0.05 (that is the statistical significant value).*

tight long-term clinical and echocardiographic surveillance of these patients. However, the choice of surveillance modalities and recommendations for interventions are frequently based on expert consensus rather than trial data and depends upon local expertise and availability (30–32).

A recently published harmonization of international guidelines made an effort to provide surveillance recommendations for survivors of CCSs by an evidence-based approach and identifying subgroups at increased risk of developing cardiomyopathy (33).

Repeated echocardiographic assessment of LVEF remains the major way to identify a subclinical impairment of cardiac function. Conventional echocardiographic measurements of LV function, however, are not always adequate to detect early stages of cardiomyopathy (4, 9, 33). Importantly, this might be achieved using some new echocardiographic methods, that have been shown to be more sensitive at identifying early impairment of LV function (10).

The aim of this study was to assess which non-invasive echocardiography-based functional cardiac measures can detect early subclinical myocardial changes in a cohort of CCSs afferent to our institution.

Two-Dimensional Echocardiography and Tissue Doppler Imaging

Traditionally, monitoring of anthracycline-related cardiotoxicity has relied on serial 2D echocardiography utilizing LVSF and LVEF (24, 34). However, these 2D Echo parameters are load-dependent, demonstrate intra-patient and inter-observer variability, and might not detect more subtle changes in cardiac systolic function (4, 9, 33). Our current study demonstrated significantly decreased LVSF in CCSs compared to healthy controls ($p = 0.027$), but no significant difference in LVEF, with all CCSs having LVSF and LVEF values within the normal range. Our findings are similar to those found in a recent multicenter cohort study by Slieker et al. (35), demonstrating lower measures of global systolic function in CCSs when compared with healthy controls; however, these differences were small and parameters of LV function were largely within the normal range. In contrast, another recent study by Border et al. (36) found a measurable longitudinal decline in many standard echocardiographic parameters of cardiac function, including FS and EF, in CCSs compared to controls, supporting the utility of conventional measures of global systolic function to detect myocardial dysfunction in cancer population.

These discordant results could be explained by differences in age and follow-up duration of CCSs across studies. For example, we investigated younger children compared to those of Border's study, and this can be relevant, as it is well-known that advanced age and longer off-therapy time are important risk factors for the detection of more advanced disease. Therefore, two-dimensional LVEF likely may identify late stages of systolic dysfunction, whereas more sensitive methods are need for early detection of subclinical abnormalities of LV function.

An echo parameter designed to identify early myocardial disease is the MPI, which incorporates measures of systolic function (IVCT and LVET) and diastolic function (IVRT) and is independent of HR, ventricular geometry and preload/afterload (11). Previous reports supported the utility of MPI as an early marker of subclinical LV dysfunction in the surveillance of cardiotoxicity (34, 36–38). In our study, global myocardial function evaluated through MPI resulted significantly lower in cases than in controls ($p = 0.005$), with IVCT mildly increased ($p = 0.04$) and without significant differences in LVET and IVRT measures. At present time, even though MPI has been reported to identify early myocardial dysfunction, its clinical utility and prognostic value in progression of late anthracycline cardiomyopathy remains still unclear.

As a decrease in diastolic function often precedes the decline in systolic function (39), we investigated whether diastolic function assessment may provide a useful early marker of cardiac disease in CCSs patients. Diastolic dysfunction has been reported in several studies in CCSs exposed to anthracycline therapy (34, 36, 38, 40, 41) although Harahsheh et al. found normal diastolic function in these patients (42). In our study, we found mildly decreased E/A ratio in CCSs compared to controls ($p = 0.046$), but all diastolic parameters measured by pulsed wave Doppler and TDI were normal, without any significant difference between both groups. We observed an inverse correlation between the E/A ratio and the cumulative anthracycline dose. In clinical practice, surveillance of CCSs should include measures of diastolic function, although the utility of diastolic parameters to predict subsequent anthracycline-related cardiotoxicity warrants further follow-up studies.

Speckle Tracking Imaging and 3D Echocardiography

Recently, myocardial strain imaging obtained by STE and 3D echo have been suggested to be more sensitive techniques than standard echocardiography for the earlier detection of subtle changes in myocardial function after anthracycline therapy (43). Recent reports from international Cardiological Societies recommend measurements of LV function by STE and 3D Echo in the long-term follow-up of adult cancer survivors (29, 44), but their role in pediatric CCSs has not been well established (33). Therefore, these advanced echocardiographic techniques require further validation with longitudinal follow-up studies, prior to be recommended in clinical practice, dependent also on availability and adequate operator experience (33, 35, 45).

Myocardial strain imaging, obtained by STE, evaluates the change in myocardial fiber length as compared to its original length in the plane in which it is measured (15). Unlike conventional LVSF and LVEF, which are limited to one plane, strain can be measured in three planes, providing a better determination of cardiac function. Previous studies provided evidence that strain abnormalities can be seen early despite preserved LVEF by conventional echo

measures, identifying cardiac involvement in CCSs prior to the development of overt systolic dysfunction (43, 46–54). However, a more recent multicenter study of cardiac strain in a large cohort of pediatric CCSs found only mild differences in parameters of longitudinal strain between patients and healthy controls, with most CCSs (92.3%) having longitudinal strain values within the normal range. Thus, this report concluded that the utility of strain imaging in the long-term follow-up of CCSs remains to be demonstrated (35). Our study found normal values of global strain in both groups, and there was no significant difference in peak mean systolic longitudinal strain in any apical view. Furthermore, when stratifying for each segment, no difference was found in terms of regional longitudinal strain.

Among newer techniques, 3D echo has been shown to overcome the geometric limitations of 2D Echo for the assessment of LV volumes and EF and has been validated against CMR in assessing the LV volumes and EF in both children (17) and adults (18). In studies performed in survivors of childhood (9) and adult-onset cancer (55), 3D Echo demonstrated the lowest inter-observer and serial variability for measurement of LV systolic function, increasing the accuracy of detecting more subtle changes in LVEF. In this study, we found significant lower measures of three-dimensional LVEF in CCSs compared with healthy controls ($p = 0.002$), even though values were largely within the normal range. This finding of lower LVEF among CCSs using 3D Echo, agrees with several previous studies (9, 43, 47, 56, 57). Furthermore, several previously published studies found 3D Echo to have the highest sensitivity to detect more abnormalities in the ventricular function than conventional echocardiography, when compared to CMR (4, 9, 57).

In our study, we did not demonstrate statistically significant correlations between echocardiographic parameters (2D, strain and 3D assessment) and age at diagnosis or duration of follow-up. Similarly, a correlation between the echocardiographic parameters and established risk factors for anthracycline cardiomyopathy could not be documented. This may be due to a small sample size and/or young age of this cohort.

In conclusion, our study found significantly reduced 3D LVEF in CCSs compared with healthy controls, despite no significant differences in 2D EF and longitudinal strain values. These findings might support the use of 3D echo for the detection of early LVEF in CCSs.

CONCLUSION

In recent years, cardiac surveillance for early detection of late subclinical cardiac dysfunction in CCSs treated with anthracycline has received increasing attention. Although commonly used, conventional echocardiographic parameters may not detect early cardiovascular damage in these patients. Novel imaging techniques, including ST imaging and 3D echo, have been investigated, but further longitudinal studies are necessary to clarify and optimize their role in routine clinical practice. The challenge with these non-conventional methods is to understand whether an abnormal result may be considered predictive of subsequent overt LV dysfunction, which might allow the identification of patients at risk and undertaking early preventive strategies.

Limitations

Some limitations of our study should be acknowledged. First, we did not perform a preliminary sample size calculation to plan the number of subjects to enroll in the study. However, since previous studies showed that CCSs may have a decrease in LVEF of 10% (58, 59), we calculated that 6 subjects per group only might have been sufficient to have an 80% statistical power to detect as significant at two-tailed $p < 0.05$ a reduction of 10% in LVEF in CCS compared to healthy controls (LVEF 65.3 ± 4.4%). However, larger population may be required to better define the entity of LV impairment in CCSs. Second, CCSs were included among those referred to our University Center and therefore they may not reflect the general population of children treated with anthracyclines. Finally, the follow-up of our patients was done at 6 years from treatment and therefore we cannot exclude that some different results may be found at longer term.

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 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

Conceptualization, AnR, AD, AL, and GL. Methodology, PL, VM, and AV. Writing—original draft preparation, AlR, RS, LB, and GA. Writing—review and editing, AD, RS and AL. Supervision, AnR and GL. Statistical analysis, GL, GA, and AV. All authors contributed to the article and approved the submitted version.

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OPINION
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Digital Phenotyping and Dynamic Monitoring of Adolescents Treated for Cancer to Guide Intervention: Embracing a New Era

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INTRODUCTION

Adolescents diagnosed with and treated for cancer represent a particular vulnerable patient population with unique and complex medical and psychosocial needs that extend beyond the completion of treatment (1–4). These cancer patients are in the most critical phases of their development (5–7), with brain, body, mind, and social environment in constant transformation conferring vulnerability to some individuals and resilience to others. Adolescence is characterized by a heightened incidence (and first onset) of various mental illnesses (8–10). Cancer during adolescence influences peer relationships, changes mood and behavior, interferes with cognitive functioning, educational achievement, and increases the risk for the onset of psychopathology (11, 12). Furthermore, treatment is often accompanied by numerous physical and psychological symptoms such as nausea, vomiting, sleep disturbance, fatigue, pain, changes in body weight and body-image (2), treatment-induced psychosis, depression, and anxiety. Importantly, peer interactions in young cancer patients is often suddenly interrupted putting them at a higher risk for the development of emotional and behavioral problems. Even though many of these concerns remain largely unattended because of a lack of timely detection and management (13, 14), recent data indicate a growing awareness of the specific needs of this patient population suggesting that a concerted effort should be made to develop age-appropriate resources that could help them manage their illness and its treatment (15).

When asked, adolescents indicate five domains of concern: 1) physical, emotional, behavioral, and mood problems related to treatment; 2) psychosocial issues; 3) present and future adjustments once the therapy has finished; 4) transition, organization, and management of survivorship; and 5) need to be better connected, involved, and informed (16–20).

Given this, it is vital to capture, as early as possible, the biological, psychological, emotional, behavioral, and social modifications in young cancer patients to expand our day-to-day understanding of the factors affecting their vulnerability.

Most importantly, reviews and guidelines based on best practice stress the fact that cancer in adolescents does not always have chronic negative outcome especially if we understand which individuals are at heightened risk (20). Any issue in the five domains of concern listed above must be detected early to put timely and preventive measures into place (21). Adolescent cancer patients represent an enormously heterogenous group and while most somatic treatments are accompanied by the development of acute or chronic unwanted effects (2, 4), we currently lack the capacity to distinguish individual trajectories leading to increased vulnerability or resilience. A paradigm shift is essential to detect the distinct clinical pathways of high-risk individuals (22–24).

Current innovative digital approaches to patient care and monitoring offer a unique opportunity to create predictive models of individual vulnerability based on the integration and interdependencies of diverse sources of information. The analysis of the data contained in these platforms allow the projection of potential outcomes and disease trajectories, identifying those patients that are progressing from generic vulnerability to becoming at high risk for a negative event.

Digital tools may have numerous potential benefits compared to traditional assessments: they are non-invasive, ecological, do not demand extra efforts, and provide continuous access which offers timely understandings of the emotional, behavioral, cognitive, and treatment related changes. Also, while not all studies underline a clear benefit, some of them indicate that they are unrestricted by time and place, and offer immediate access to data and intermediate endpoints, reduce stressful visits, remove barriers to access to care (fear, isolation), stimulate patient empowerment, and, most importantly, help in the identification of high-risk patients and their risk stratification (23–26). As a result, the integration of various digital tools in a "toolbox," would offer a concrete opportunity to modify, replace, or accompany the current more categorical approach and shift to a multimodal dimensional methodology (27, 28) in which evidence is gathered from different domains, ranging from subtle neurocognitive disfunction (29–33) to biomarkers. Ultimately, the multidimensional continuous recording of data and especially their dynamic relationships would become an integral part of the care plan and serve as specifiers of an high-risk status (34, 35).

A DIGITAL APPROACH IN UNDERSTANDING ACUTE AND CHRONIC BEHAVIORAL TOXICITY IN ADOLESCENTS TREATED FOR CANCER

Progress in the use of digital technologies and data analytics have created unmatched prospects to evaluate and alter health behavior and outcomes. Young adults and adolescents display a high compliance with the digital world surrounding them and are very comfortable with modern technologies (36, 37). However, while acceptance of digital tools is high (19), many health applications and tools are not specific for adolescents. Apps for various pathological conditions exist, supporting the life of patients suffering from diabetes, obesity, hypertension, and psychopathology (38). Several apps, mostly focused on symptom tracking and monitoring quality of life, exist for adult cancer patients while very few have been developed with the adolescent patient in mind (25, 39–44).

DIGITAL PHENOTYPING AS PART OF A DIGITAL TOOLBOX

Adolescents with cancer consider psychological, emotional, cognitive, and social problems, issues of major concern (11–13). Touching the right cords is fundamental if we want to protect and help them. Digital phenotyping offers promising features for use in this patient group because it may allow objective and continuous measurements, documenting, and quantifying mood, energy level, and cognition in a non-invasive manner using personal digital devices (such as smartphones or wearables). This ecological approach centered on patients' everyday lives has proven valuable when assessing symptomatology. Analysis by real-time algorithms allows for checking and detecting alarming pattern changes in daily functioning and predict when an individual shifts from being at risk to a patient that is about to experience an episode in need of immediate care (32, 33). Thus, digital phenotyping provides a period of long continuous surveillance of disease related moderators and mediators elucidating the temporal dynamics between specific biological mechanisms and changes in mood and behaviors.

Digital phenotyping, however, should be taken as just one of the instruments of a digital health toolbox. If we want a comprehensive approach, digital phenotyping must be accompanied by other participatory and communication tools, which include various objective biological markers that can be detected by sensors, wearables, and devices, such as smartwatches, rings, and fitness trackers (38, 45–47). Digital biomarkers and dynamic monitoring of functions, such as heartbeat, weight, blood pressure, temperature, sleep patterns, fatigue, and pain, provide important additional parameters related to disease, treatment related side effects, and effectiveness of treatment.

Lastly, the toolbox should also include the use of subjective measures, such as patient reported outcomes since they capture different nonobjective aspects of the same clinical construct, for instance fatigue (22, 25, 44). This information requires the active participation of the individual including symptom reporting or the use of activity diaries tracking personal impressions and perceptions, or responding to periodic questionnaires related to personal wellbeing and psychosocial health. A final advantage of an integrated digital approach is putting the adolescents at the very center of the data generation to fulfill their much-felt need to be informed, educated, and connected (20). In addition, providing access to information, videos, tutorials offering access to support groups or patient advocacy organizations (if desired) will connect and empower them even more.

Digital phenotypes, digital biomarkers, mobile asynchronous questionnaires, and self-reports provide data along various dimensions that, once collected, will reflect the complexity and delicacy of the behaviors of adolescent patients. Challenges also exist with this approach since to benefit from a digital analysis, very large amount of data reflecting the complexity of developmental and disease related factors that may alter individual behavior almost instantly must be factored in (**Figure 1**).

DYNAMIC MAPPING OF MULTIDIMENSIONAL DATA

At present, evidence has accumulated that the engrained traditional approach linking just one or a few (often molecular or biological) mediators to an illness related phenotype, limits the understanding of the complex underlying pathologic conditions which has hampered progress in the development of efficient treatments.

To this end, traditional statistical approaches are not capable of capturing the particularity of each individual specific phenotype. At present, modern approaches coming from machine learning, especially those related to deep learning, provide innovative ways to tackle the complexities of multidimensional continuous data collection. The integration of data collected from the different instruments in a digital toolbox, ranging from sensor recorded physical functioning to digital phenotyping, need a more sophisticated approach, such as deep learning and network analysis. Recently, researchers have started to use deep neural networks in big-data rich areas such as online social media platforms or smartphone and mobile sensor-based data relating them to mental health (48–51).

Therefore, combining network science and dynamic system theory with a toolbox containing digital phenotyping and biomarkers permits the integration of data across diverse levels of analysis and capture the nature of their dynamic relationship over time, both for patients and treatment modalities (52–57) (**Figure 1**).

New conceptual thinking may result in increasingly explanatory and predictive models resulting in a more realistic image of the strengths and vulnerabilities of these young patients at high-risk for behavioral and emotional problems. Network analysis helps to determine if one domain or function is more important than others, if changes in one domain, factor, or symptom dynamically influence the function of others, and finally, what factor or domain is driving the network. In sum, network analysis will not only indicate which domains and factors mostly define individual vulnerability but will have important implications for clinical practice motivating personalized strategies in the prevention of mood, behavioral, and cognitive problems (46, 54–57).

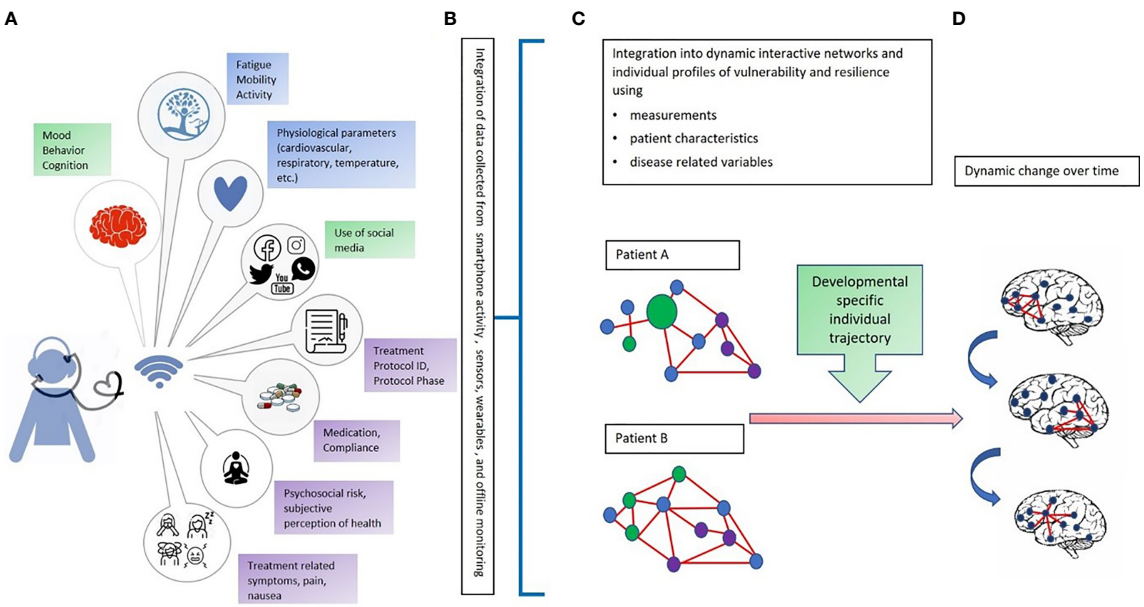

FIGURE 1 | An integrated multi-dimensional model for the use of digital phenotyping as part of a digital "toolbox" to be used in adolescents with cancer. Our hypothetical model is defined over four phases: **(A)** The data collection phase: In green: Objective continuous non-invasive data collection related to mood, behavior, cognition, and use of social media (using a digital phenotyping app); in blue: Objective continuous non-invasive collection of biomarkers from sensors and wearables (heartrate, blood pressure, temperature); in purple: Periodic collection of subjective perceptions and impressions related to mental and physical wellbeing (e.g., diaries, self-reports, and questionnaires) and type of treatment, protocol, and protocol phase; **(B)** The data transformation phase, that is, the conversion of raw data into usable algorithms and outputs to feed data analysis and network construction; **(C)** Creation of individual networks based on data from different dimensions and domains to understand the strengths and needs of each patient. The circles represent signs, symptoms, and factors related to the domains of data collection (green, blue, and purple). The red lines represent the connections among signs, symptoms, and factors; **(D)** Development of dynamic networks to monitor change over time in network organization and connectivity aimed at identifying windows of vulnerability and resilience. Letters and colors refer to those shown in the figure.

INEVITABLE OPPORTUNITIES, INEVITABLE CHALLENGES

Notwithstanding the promise to deliver a revolution in the health care of children and especially adolescents diagnosed with cancer, the incredible potential of the digital era comes with substantial questions regarding its implementation.

While artificial intelligence, deep learning, and network analyses sustain the analysis of complex data, their use requires the acquisition of innovative expertise and competence. The analysis of large multi-domain data bases needs a multidisciplinary collaborative effort which is currently lacking.

Today, more than in the past we are witnessing an increasing number of disruptive products such as advanced mRNA, gene and cell therapies that face unexpected legal and ethical challenges. Therefore, data must be managed in a thoughtful way. We must prepare for a profound change in the way data are gathered and integrated from multiple sources including digital tools. For digitally captured data, quality measures must be incorporated, and the sensitivity, specificity, accuracy, and precision of device parameters and measurements need to be tested.

Also, personal health-related data, especially from minors, should be considered in a meaningful way. If we want to engage young patients as data generators, a trusted ethical, legal, and regulatory ecosystem should be created with clear rules. Innovative, legal, organizational, and technical solutions are needed to share their sensitive, health-related personal data in an ethical and privacy-compliant environment.

Therefore, adopting this novel approach should be accompanied by adequate ethical and regulatory support to fully protect the patient and to overcome present and future challenges linked to confidentiality and accountability. While having the knowledge and the technical capacity to truly benefit from digital tools, widespread information, education, and training would be needed from both the clinician and the patient. Thus, we, adults, clinicians, and researchers need to step up to the challenge and avoid becoming the rate limiting step in embracing a new era (23, 24).

POSSIBLE IMPACT

The impact of digital phenotyping and dynamic monitoring likely involves multiple domains of care as well as multiple stakeholders. Researchers will have to find new ways to enrich, share, and analyze data and develop strategies to support patient friendly access. At the same time research should direct efforts to the development of decision-making paradigms that better inform and sustain clinical interventions. For patients, the use of a digital toolbox represents an innovative way to study their needs and challenges while being treated for cancer. With the patient as active participants we will be better able to transform their needs into interventions that aim to support their wellbeing and prevent serious problems before they start. Fine-grained phenotypes representing the individual aspects of emerging problems may induce industry to develop new therapies and sustain faster progress from bench to bedside. Finally, an ecological day-to-day collection of data should stimulate regulators to identify new targets and determine when and how a target may become acceptable for regulatory decision making centered on the adolescent.

WITH THE FUTURE OF THE ADOLESCENT PATIENT IN MIND

The care process surrounding the adolescent patients is an active, planned, coordinated, comprehensive, multidisciplinary, and multi-stakeholder process that should be flexible, developmentally appropriate considering the interaction and interdependency of medical, psychosocial, behavioral, and environmental factors. The use a digital toolbox combined with network analysis may define developmental trajectories of risk and resilience and reveal more fine-grained cognitive and behavioral phenotypes, that together with clinical factors, and biological markers, may explain the relationship between disease, age specific risk, and the efficacy of treatment. To achieve this, the combination of sufficiently diverse tools, by themselves not new, will feed complex datasets with broad as well as deep phenotypic representation of the patients' needs.

THE ADDED VALUE OF ENTERING THE DIGITAL ERA

According to the suggested paradigm shift, each individual young patient will be involved in a process aimed to predict, prevent, and personalize their care, stressing the individual's participation moving from a passive receiver of care to an active and conscious contributor to their wellbeing.

A "digital toolbox" which includes digital phenotyping will give us hope for the future (58) and will help to enrich the care of adolescent patients with cancer. Starting from each individual patient, and within known treatment modalities and protocols, we will be better able to recognize interrelated behaviors, risk factors, and treatment and disease related side effects and to isolate the domain(s) most central to possible adverse outcome. Combining pharmacovigilance and behavioral phenotyping with easily accessible technologies and the most innovative analyses will improve timely access to the detection of adverse medical and behavioral events. In turn, this will stimulate efforts to improve, develop, and implement innovative programs of personalized interventions adapted to the clinical needs and characteristics of individual patients. Finally, such a process, would fully respect the so-called P4 of Precision Medicine: predictive, preventive, personalized, and participatory, putting the adolescent patient at the very core of their present and future health.

AUTHOR CONTRIBUTIONS

JMCB and LP conceived the manuscript. JMCB wrote the first draft. LP, CB, CC, and FT critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Cisplatin Ototoxicity: Examination of the Impact of Dosing, Infusion Times, and Schedules In Pediatric Cancer Patients

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Background: Sensorineural hearing loss is a well-known side effect of cisplatin (CDDP). There is limited research on the effect of dosing, infusion times, and schedules of cisplatin administration and their impact on hearing loss.

Methods: A retrospective review of 993 pediatric patients' medical and audiological charts from August 1990 to March 2015 was conducted using stringent inclusion criteria to characterize patients with hearing loss. 248 of these patients received CDDP. Of these, 216 patients had sufficient CDDP infusion data to assess for sensorineural hearing loss attributable to CDDP and its associated risk factors. Chart reviews were performed to extract clinical data including CDDP dosing information. Demographic and clinical characteristics were summarized by descriptive statistics, and univariate and multivariate logistic regressions were performed to examine the relationship between hearing loss and specific parameters of cisplatin administration (amount infused per dose, prescribed infusion time, total number of doses, number of doses per cycle, number of cycles, cumulative cisplatin exposure). Stepwise variable selection procedure was performed in the multivariate model building to extract the best subset of risk factors for the prediction of hearing loss and worsening ototoxicity grade using an established ototoxicity grading scale from the International Society of Pediatric Oncology (SIOP).

Results: A total of 153 patients with complete medical and audiologic data were evaluable for analysis. Hearing loss was identified in 72.6% of the patients. Multivariate analysis revealed that age [OR=0.90 (0.84-0.97), *p*-value=0.0086], radiation to any part of the body, [OR=3.20 (1.29-7.93), *p*-value=0.012], amount infused per dose (mg/m^2) [OR=1.018 (1.002-1.033), *p*-value=0.029], and cumulative cisplatin exposure (mg/m^2) [OR=1.004 (1-1.008), *p*-value=0.027] were associated with hearing loss. Similar associations were also found between these risk factors and worsening SIOP grade.

Conclusion: In one of the largest studies examining the influence of CDDP dosing and schedules on hearing loss, we found the amount of CDDP infused per dose is a significant

risk factor. Considerations in designing regimens that reduce the amount of CDDP infused per dose may reduce the risk of hearing loss. Randomized prospective trials are needed.

Keywords: cisplatin, pediatric, cancer, ototoxicity, dosing

INTRODUCTION

Sensorineural hearing loss (SNHL) is a well-known complication from the administration of cisplatin (CDDP) (1–6). Evidence suggests there is long-term retention of CDDP in the cochlea, and a dose-dependent relationship between a higher cumulative dose and a higher incidence of hearing loss has been established (7–9). Hearing loss may evolve during therapy, after its completion, or may not even present until years following the end of treatment (5, 10–12). Though the relationship between hearing loss and CDDP has been thoroughly examined, the effect of variables related to CDDP dosing and administration (the amount per dose, frequency, and dosing schedules) and their relationship to hearing loss have not been well-defined. This lack of knowledge limits our ability to establish strategies for reducing ototoxicity through modification of dosing parameters (4, 13, 14). Due to the limited research on this topic to date, we sought to examine the relationship between parameters of CDDP dosage or administration and the presence of hearing loss in a cohort of pediatric cancer survivors.

PATIENTS AND METHODS

This study, approved by the Institutional Review Board of the Washington University School of Medicine Human Research Protection Office, was a retrospective chart review of medical record data existing at the initiation of our study. Audiology charts of pediatric oncology patients at St. Louis Children's Hospital treated from August 1, 1990 through March 31, 2015 were reviewed. From this cohort, patients were assessed for treatment containing CDDP.

Inclusion criteria into our current study required prior CDDP treatment. Patients whose chemotherapy treatment did not include CDDP were excluded. Evaluable patients had either completed their CDDP therapy or had documented CDDP hearing loss from current therapy and were still being treated at the cutoff for study entry in March 2015. Data was not collected for patients still undergoing treatment who had normal hearing at the cutoff point of the study. No patients received oto-protectants. The medical records of these patients were reviewed to extract the following variables of interest: gender, birthdate, date of diagnosis, race, ethnicity, diagnosis, CDDP dosage information (i.e. cumulative dose, number of doses, amount per dose, doses per cycle, dosage time, dosage reduction), presence of carboplatin, radiation exposure to any part of body, radiation exposure to head, date when all therapy ended, date of last CDDP administration, living status, date of most recent audiogram, presence of hearing loss based on the worse ear, and right/left ear toxicity grades according to the International Society of Pediatric Oncology (SIOP) (15). Each patient's chart was individually evaluated for the specific variables mentioned above; the actual dosing of CDDP recorded in the medical record was used, and there was no imputed data regarding the amount of CDDP the patient received to ensure that the dosage information is patient specific. We were unable to obtain consistent and specific documentation of the infusion times for CDDP. Infusion times utilized were the prescribed infusion times derived from the patient's orders or treatment plan. After review, thirty-two patients were excluded as specific CDDP dosage information was not available, resulting in a cohort of 216 patients eligible for analysis in the current study.

A substantial effort was made to ensure clear audiologic data. To uphold the audiologic parameters utilized in our previously reported investigations, we adhered to formerly established criterion to ensure the study subjects had treatment acquired, ear specific, sensorineural hearing loss (16). This resulted in the exclusion of many patients but created a pediatric population with less ambiguous hearing profiles and allowed for a more rigorous investigation into the presence of ototoxic hearing loss in this population. The following details the stringent audiologic parameters required for inclusion in this study.

Baseline Audiograms

All audiograms included in our analysis were of good to fair reliability, as determined by the testing audiologist. Soundfield and ABR testing was allowed for baseline testing. All patients were required to have a normal baseline audiogram with a subsequent ear specific behavioral audiogram, testing out to 6000 and/or 8000 Hz; baseline audiograms obtained *via* soundfield testing were included as long as the subsequent hearing test revealed ear specific thresholds. According to our institutional standards, a normal behavioral hearing test was defined as thresholds of ≤ 20 dB HL from 1 kHz - 4 kHz and ≤ 30 dB HL at 6 kHz and 8 kHz, in order to account for tympanostomy tubes and collapsing canals from earphones. A normal auditory brainstem response (ABR) at our institution is defined as thresholds of ≤ 30 dBnHL from 0.5 kHz -1 kHz and ≤ 20 dBnHL at 2 kHz - 8 kHz, using both earphone and inserts as transducers.

All evaluable baseline hearing tests required at least 2 thresholds from 1 kHz - 4 kHz. If any frequency was outside the defined normal range, from 1kHz - 4kHz, the audiogram for that ear was not evaluable. Ears with conductive hearing loss ≥ 1000 Hz at baseline were excluded. Patients were included in our analysis if they presented with normal hearing in at least one ear at baseline. In this case, audiologic data was only collected on the single, normal hearing ear. Finally, our study allowed the baseline audiogram to be absent if a subsequent audiogram documented normal hearing.

Most Recent Audiograms

All audiograms evaluated as the "most recent audiogram" were behavioral hearing tests; soundfield alone and ABR testing were not permissible. All sensorineural hearing loss ≤ 4 kHz, were confirmed by bone conduction thresholds. Bone is not tested above 4 kHz at our institution. If the hearing loss was observed at ≥ 6 kHz, documentation of a normal middle ear status through static admittance of ≥ 0.3 mmho, large ear canal volume consistent with patent tubes, and/or a normal otologic exam by an otolaryngologist was needed to confirm sensorineural nature of the hearing loss. In the current study, the presence of normal bone conduction thresholds and/or a normal hearing evaluation at both baseline and at the most recent hearing test superseded abnormal tympanometric measures. The patient was determined to have hearing loss if the most recent audiogram showed change from normal baseline and a SIOP grade of greater than 0 in the worse evaluable ear.

Each evaluable ear was assigned a SIOP grade relative to the bone conduction thresholds of the most recent audiogram. The SIOP grading scale has been suggested as the superior grading scale in regards to classifying ototoxic hearing loss (17, 18). Audiograms with a normal 4 kHz threshold but an absent or an unevaluable 6 kHz and 8 kHz threshold were codified as "not gradable test", meaning that it could not utilize the SIOP classification. For this study, SIOP grades 1 and 3 were based on at least one obtained frequency referenced in the SIOP grade level (6 or 8 kHz in grade 1 and 2 or 3 kHz in grade 3). Only 4 patients with hearing loss were not assigned a SIOP grade, as they were still undergoing treatment. Assignment of a SIOP grade to a patient with ongoing treatment would not accurately represent the total ototoxic damage that may develop once the patient's CDDP treatment is completed.

Statistical Methods

Study data were collected and managed using REDCap electronic data capture tools hosted at Washington University School of Medicine (19, 20). REDCap (Research Electronic Data Capture) is a secure, web-based software platform designed to support data capture for research studies, providing 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for data integration and interoperability with external sources

All statistical analyses were carried out using SAS version 9.4 (SAS Institute, Cary, NC). Demographic and clinical characteristics were summarized by descriptive statistics, i.e., median and IQR (interquartile range) for continuous variables; and count and percentage for categorical variables. Group differences were examined by Kruskal-Wallis test for continuous variables and Fisher's exact test (if cell count less than 5) or Chi-square test for categorical variables. Univariate and multivariate logistic regression were used to examine the relationship between hearing loss/SIOP grade and various risk factors, including sex, race, diagnosis, carboplatin use, radiation, radiation to the head, age, total number of doses, the amount of CDDP infused per dose, number of doses/cycle, number of cycles, prescribed dose time, and cumulative CDDP dose. A stepwise variable selection procedure was performed in the multivariate model building for the outcome measure, hearing loss. A significance level of 0.3 was required to allow a risk factor to enter or stay in a model during the variable selection process. To make the results comparable, the final multivariate risk model of hearing loss that obtained during the stepwise selection was used as the multivariate risk model for the outcome measure, SIOP grade.

RESULTS

There were 993 patients in the entire cohort. After assessing their treatment, 248 patients were included in the study who had a prior history of CDDP exposure (**Figure 1**). Thirty-two patients were excluded as specific CDDP dosage information was not available. This resulted in a total of 216 evaluable patients for the current study. Of the total 216 patients, 153 had sufficient audiometric data necessary to assess their hearing status.

Table 1 summarizes the patient and treatment characteristics based on the presence or absence of ototoxic hearing loss. The demographics of patients included in this study are representative of the general pediatric cancer population treated at St. Louis Children's Hospital – predominately Caucasian with a slight male predominance. The diagnoses of patients in this study reflect patient populations that undergo treatment regimens containing CDDP. There were 111 patients (72.55%) with hearing loss, and 42 (27.45%) with normal hearing. Eight-five (55.6%) of patients were male, and 68 (44.4%) were female. The average age of our patient population was 8.3 years old, ranging from 0.2 to 19.9 years of age. The mean time in years from the last CDDP administration to the last recorded audiogram was 3.73 years (standard deviation= ± 3.77 years). Thirty-one patients (20.3%) were treated with carboplatin in addition to CDDP. There were 91 patients (59.5%) who received radiation, with 65 patients (42.5%) receiving radiation to the head. The mean CDDP cumulative dose of 391.2 mg/m ^{2}, ranging from 90 to 1000 mg/m ^{2}. The mean amount per dose was 74.1 mg/m ^{2}, ranging from 20 to 150 mg/m ^{2}. Patients received an average of 6.9 doses (range=1-30) and 3.7 cycles (range=1-8) with an average of 2.1 dose per cycle (range= 1-9). The average prescribed dose time was 4.4 hours, (range= 1-8).

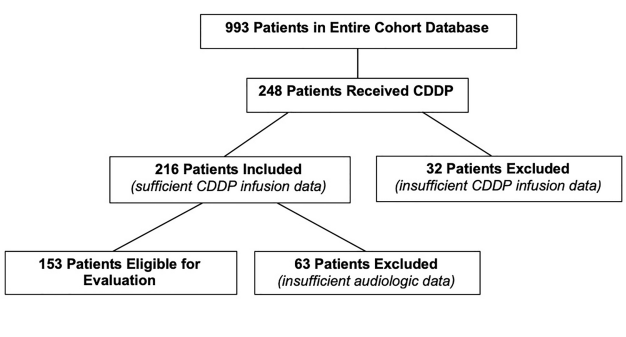

FIGURE 1 | CONSORT diagram outlining selection of eligible patients.

TABLE 1 | Patient and treatment characteristics by the presence of hearing loss.

Characteristics[b]	Presence of Hearing Loss[a]			
	Total	Yes	No	p^c
No. of Patient	153 (100%)	111 (72.55%)	42 (27.45%)	
SIOP Grade				
0	41 (27.9%)	0 (0%)	41 (100%)	
1-2	55 (37.4%)	55 (51.9%)	0 (0%)	
3-4	51 (34.7%)	51 (48.1%)	0 (0%)	
Age (Years)	153, 7.8 (3-13.3)	111, 6.1 (2.6-11.2)	42, 13.1 (7.2-16.2)	0.0003
Sex				0.3949
Male	85 (55.6%)	64 (57.7%)	21 (50%)	
Female	68 (44.4%)	47 (42.3%)	21 (50%)	
Race				0.9819
White	126 (83.4%)	91 (83.5%)	35 (83.3%)	
Non-White	25 (16.6%)	18 (16.5%)	7 (16.7%)	
Diagnosis				0.0013
Medulloblastoma	42 (27.5%)	35 (31.5%)	7 (16.7%)	
Non- Medulloblastoma brain tumor	21 (13.7%)	18 (16.2%)	3 (7.1%)	
Neuroblastoma	24 (15.7%)	21 (18.9%)	3 (7.1%)	
Osteosarcoma	32 (20.9%)	15 (13.5%)	17 (40.5%)	
Other	34 (22.2%)	22 (19.8%)	12 (28.6%)	
Carboplatin Use				0.4962
Yes	31 (20.3%)	24 (21.6%)	7 (16.7%)	
No	122 (79.7%)	87 (78.4%)	35 (83.3%)	
Radiation				0.0100
Yes	91 (59.5%)	73 (65.8%)	18 (42.9%)	
No	62 (40.5%)	38 (34.2%)	24 (57.1%)	
Radiation to the Head				0.0121
Yes	65 (42.5%)	54 (48.6%)	11 (26.2%)	
No	88 (57.5%)	57 (51.4%)	31 (73.8%)	
Total Number of Doses	147, 6 (4-8)	107, 6 (4-8)	40, 6 (4-8)	0.2614
Amount of CDDP Infused Per Dose (mg/m2)	147, 75 (50-90)	107, 75 (50-100)	40, 60 (55-75)	0.0193
Number of Doses/Cycle	146, 1 (1-3)	106, 1 (1-4)	40, 2 (1-2.5)	0.0482
Number of Cycles	146, 4 (2-5)	106, 4 (2-5)	40, 4 (2-4.5)	0.8423
Prescribed Dose Time (hours)	122, 6 (4-6)	86, 6 (1.5-6)	36, 4 (4-6)	0.2135
Cumulative CDDP Dose (mg/m2)	153, 400 (300-480)	111, 400 (300-480)	42, 383.5 (299.7-480)	0.3272

[a] *Hearing loss defined by > 0 SIOP grade.*
[b] *For categorical variables, (n (%)) is reported, excluding missing values. For continuous variables, "n, median (Q1-Q3)" is reported, where Q1 is 25th percentile, and Q3 is 75th percentile.*
[c] *Fisher's exact test (if cell count less than 5) or Chi-square test for categorical variable; Kruskal-Wallis test for continuous variable.*

Hearing Loss

Analysis of evaluable patients revealed a difference in hearing loss frequency linked to age (p=0.0003), diagnosis (p= 0.0013), radiation to any part of the body (p=0.010), radiation to the head (p=0.012), amount of CDDP per dose (p=0.019), and number of doses per cycle (p=0.048) (**Table 1**). Univariate binary logistic regression analyses demonstrated that hearing loss was associated with age (p= 0.0002), diagnosis (p=.0021), radiation to any part of the body (p=0.011), radiation to the head (p=0.014) and amount of CDDP per dose (p=0.01) (**Table 2**). Multivariate binary logistic regression revealed younger age [OR=0.90 (0.84-0.97), p = 0.0086], radiation to any part of the body [OR= 3.2 (1.3-7.9), p=0.012], larger amount of CDDP per dose [OR=1.018 (1.002-1.033), p=0.029], and larger cumulative CDDP dose [OR=1.004 (1-1.008), p=0.027)] significantly increased the risk of hearing loss (**Table 2**).

SIOP Grade

Univariate and multivariate multinomial logistic regression analyses were also completed to determine associated risk factors with SIOP grade (**Table 3**). Forty-one (27.9%) individuals had a SIOP grade of 0, consistent with normal hearing. Fifty-five (37.4%) of patients had a SIOP grade of 1-2, and 51 (34.7%) had a SIOP grade of 3-4. Worsening SIOP grade was associated with age (p<0.0001), diagnosis (p=0.011), radiation to any part of the body (p=0.022), radiation to the head (p=0.042), and amount of CDDP per dose (0.0093) on univariate analysis. Like hearing loss, worsening SIOP grade was associated with age (p <0.0001), radiation to any part of the body (p=0.023), and amount of CDDP per dose (p=0.037) on multivariate analysis, with cumulative dosing trending toward significance (0.057) (**Table 3**).

DISCUSSION

Increasing our understanding of the parameters of CDDP infusion effects on hearing loss can lead to strategies that may reduce the risk. This study has demonstrated the amount of CDDP infused per dose was strongly associated with an

TABLE 2 | Univariate and multivariate binary logistic regression sasessing risk factors for the presence of hearing loss.

Risk Factors	Univariate		Multivariate[a]	
	Odds Ratio (95% CI)	*p*-value	Odds Ratio (95% CI)	*p*-value
Age (Years)	0.878 (0.82-0.94)	0.0002	0.902 (0.835-0.974)	0.0086
Sex		0.3957		
Female	0.734 (0.36-1.497)			
Male	-ref-			
Race		0.9819		
Non-White	0.989 (0.38-2.573)			
White	-ref-			
Diagnosis		0.0021		
Medulloblastoma	2.727 (0.932-7.982)			
Non- Medulloblastoma brain tumor	3.273 (0.799-13.407)			
Neuroblastoma	3.818 (0.942-15.471)			
Osteosarcoma	0.481 (0.179-1.293)			
Other	-ref-			
Carboplatin Use		0.4975		
Yes	1.379 (0.545-3.492)			
No	-ref-			
Radiation		0.0111		0.0122
Yes	2.561 (1.239-5.294)		3.197 (1.289-7.933)	
No	-ref-		-ref-	
Radiation to the Head		0.0139		
Yes	2.67 (1.221-5.836)			
No	-ref-			
Total Number of Doses	0.954 (0.896-1.015)	0.1380		
Amount of CDDP Infused Per Dose (mg/m2)	1.018 (1.004-1.032)	0.0103	1.018 (1.002-1.033)	0.0287
Number of Doses/Cycle	0.91 (0.727-1.14)	0.4144		
Number of Cycles	1.031 (0.825-1.289)	0.7859		
Prescribed Dose Time (hours)	1.08 (0.902-1.294)	0.4000		
Cumulative CDDP Dose (mg/m2)	1.002 (0.999-1.004)	0.2153	1.004 (1-1.008)	0.0271

[a]*Multivariate stepwise selection results, where a significance level of 0.3 was required to allow a risk factor to enter or stay in a model during the variable selection process.*

increased risk of hearing loss; it is also associated with more severe hearing loss as reflected by a worsening SIOP grade. Other parameters such as number of doses, number of doses per cycle, number of cycles, and infusion times failed to reach significance using multivariate analysis. Cumulative CDDP was also associated with hearing loss, to a lesser degree. Prior studies have repeatedly identified cumulative incidence as a risk factor for hearing loss (4, 11, 21). The relatively modest impact of cumulative dosing of CDDP and hearing loss in this study may be due to the relative lack of variance in our cohort in their cumulative dosing (25^{th}-75^{th} percentile = 300-480 mg/m ^{2}). Thus, cumulative dosing may have been obscured as a risk factor due to the minimal difference in cumulative dosing between those with hearing loss and those without (mean = 400 mg/m^2 *vs.* 383.5 mg/m^2).

Our analysis also revealed age was strongly associated with a risk of hearing loss with the youngest patients at greatest risk. Radiation to any part of the body was also shown to be a risk factor, with radiation directly to the head failing to achieve significance in multivariate analysis. Our previous investigations had established radiation to locations other than the head still place patients at risk for hearing loss (16). Radiation can lead to circulating free radicals and inflammatory mediators that have been reported to impact organs and tissues distant from the organ targeted for radiation (ascopal effect) (22, 23). It is likely radiation to the head is also a risk factor for hearing loss given the significant correlation observed in univariate analysis. Perhaps a larger patient population would clarify its significance.

This report supports a recent study published which also identified the amount per dose as a risk factor for hearing loss (24). Our study can be distinguished from that report due to the following differences. In the aforementioned study, baseline audiograms were not required for the analysis; therefore there is no assurance whether any of the patients had a pre-existing bilateral or unilateral hearing loss prior to the initiation of CDDP therapy. We continued to utilize the rigorous criteria we have used in previous studies to ensure hearing loss in our subjects was a consequence of acquired sensorineural hearing loss, excluding ambiguous tests results. All of the subjects in our report were tested in the same audiology department, using standardized procedures and workflows with calibrated equipment in sound suites minimizing interinstitutional variance. To be evaluable, audiograms had to be of good to fair reliability with well-defined parameters to exclude patients with pre-existing hearing loss. Because our most recent audiograms required ear specific thresholds for inclusion, we were able to attribute hearing loss on the worse ear capturing the true effects of CDDP dosing on our patients. We routinely utilize the worse ear to designate hearing loss in our patients given the significant affect unilateral hearing loss can have on quality of life (25). Standard of care at our institution includes testing inter-octave thresholds when appropriate which allows for accurate SIOP grading.

TABLE 3 | Univariate and multivariate multinomial logistic regression assessing risk factors for SIOP grade.

Risk Factor	SIOP[a]					
	Univariate			Multivariate[b]		
	Odds Ratio (95% CI)		*p*	Odds Ratio (95% CI)		*p*
	Grade 1-2	Grade 3-4		Grade 1-2	Grade 3-4	
Age (Years)	0.951 (0.881-1.027)	0.765 (0.692-0.846)	<.0001	0.973 (0.89-1.062)	0.78 (0.697-0.872)	<.0001
Sex			0.6909			
Female	0.7 (0.31-1.583)	0.797 (0.349-1.819)				
Male	-ref-	-ref-				
Race			0.7766			
Non-White	1.13 (0.389-3.278)	0.773 (0.247-2.414)				
White	-ref-	-ref-				
Diagnosis			0.0108			
Medulloblastoma	1.978 (0.601-6.514)	3.429 (1.004-11.712)				
Non- Medulloblastoma brain tumor	2.462 (0.527-11.5)	4 (0.835-19.162)				
Neuroblastoma	2.462 (0.527-11.5)	5.778 (1.258-26.526)				
Osteosarcoma	0.635 (0.212-1.902)	0.167 (0.03-0.917)				
Other	-ref-	-ref-				
Carboplatin Use			0.5360			
Yes	1.079 (0.373-3.127)	1.662 (0.594-4.649)				
No	-ref-	-ref-				
Radiation			0.0223			0.0233
Yes	2.069 (0.909-4.708)	3.377 (1.413-8.069)		3.065 (1.121-8.384)	4.893 (1.437-16.653)	
No	-ref-	-ref-		-ref-	-ref-	
Radiation to the Head			0.0420			
Yes	2.273 (0.951-5.431)	3.068 (1.269-7.419)				
No	-ref-	-ref-				
Total Number of Doses	0.955 (0.888-1.027)	0.949 (0.88-1.024)	0.2994			
Amount of CDDP Infused Per Dose (mg/m2)	1.015 (1-1.031)	1.025 (1.009-1.042)	0.0093	1.019 (1.001-1.037)	1.026 (1.006-1.046)	0.0369
Number of Doses/Cycle	0.853 (0.652-1.116)	0.97 (0.753-1.25)	0.4653			
Number of Cycles	1.048 (0.815-1.348)	0.96 (0.742-1.241)	0.7674			
Prescribed Dose Time (hours)	1.094 (0.894-1.339)	1.055 (0.854-1.304)	0.6837			
Cumulative CDDP Dose (mg/m2)	1.002 (0.999-1.005)	1.002 (0.999-1.005)	0.4528	1.005 (1.001-1.009)	1.005 (1-1.009)	0.0565

[a]*for multinomial logistic regression, logits modeled use "SIOP Grade 0" as the reference category.*

[b]*to make the results comparable, the final multivariate risk model of hearing loss that obtained during the stepwise selection was used as the multivariate risk model for the outcome measure, SIOP grade.*

Our audiometric data is representative of a purely pediatric population; our mean age is older at 8.3 years. Obtaining reliable data in a young, ill child is fraught with difficulties, leading to audiograms that often fail to meet our stringent criteria for study eligibility. Many audiograms in our youngest subjects were not included in this study driving up the average age of the cohort. Finally, we included hearing loss at all levels of severity, while the recent publication only examined patients with higher SIOP grades. We had previously shown hearing loss continues to decline long after therapy is completed. We also have demonstrated that the patients with the longest follow-up had the highest likelihood of diagnosed hearing loss. Our mean time in years from the last CDDP administration to the last audiogram was 3.73 years while the previous study reported a mean of 1.5 years from the end of treatment. The increased prevalence of hearing loss as patients are followed longer and the use of the better verses worse ear may explain the difference in hearing loss observed by those investigators (43.8%) compared to our study (72.6%) (24).This late onset hearing loss particularly affects patients with established hearing loss at the completion of therapy. Thus, even mild and unilateral CDDP associated hearing loss is significant and warrants further follow-up.

This study was limited by the large fraction of patients (39%) excluded for analysis due to missing infusion or audiology data. .Ineligible patients were younger, more frequently treated with carboplatin, and were less likely to be treated with radiation. We suspect that these findings could be attributed to the differences in the number of neuroblastoma patients in the ineligible cohort (15.7% Eligible yes, 30.5% Eligible no). Neuroblastoma patients are typically younger, may receive carboplatin, and may receive less radiation due to their young age. Given the difficulties in obtaining audiograms in young patients, it is possible that young neuroblastoma patients were disproportionately excluded due to insufficient audiologic data

The study was a cross sectional analysis with no specific time point that could be used for hearing testing that could encompass the entire cohort. Audiograms were not obtained after each CDDP infusion so we could not assess any variables associated with a specific infusion encounter. However, despite these limitations and given the paucity of studies examining this subject, our audiologic monitoring program provided evidence that calls for new approaches for CDDP administration.

In order to increase the number of evaluable patients, we included patients that demonstrated SNHL, even if they had not

completed therapy. However, we did not include patients undergoing therapy if they had not demonstrated hearing loss at the end of the study period. Given that our patients were carefully screened for SNHL, which we would expect to be irreversible, we were not concerned that patients who had established SNHL would subsequently convert to the "no hearing loss" cohort with time. In contrast, there are patients who may lose hearing with subsequent cycles; this was our justification for not including those patients receiving therapy in the no hearing loss group. Furthermore, the patients with hearing loss on therapy were not included in the SIOP analysis, as it is possible the SIOP grade could shift, even while on therapy. We therefore were able to add additional patients to enhance our sample size, without including those subjects that would compromise the integrity of the cohort.

This study also collected patients over a period of almost thirty years. Although there have been advances in this field, there are no significant changes in audiology testing that would have influenced the data. However our monitoring of patients became more robust in recent years, where we now test at risk patients at regular and set intervals. Thus, the results may have been influence by the frequency of testing rather than differences in testing methods. We addressed this issue by examining the year of diagnosis (based on the date of diagnosis) as a continuous variable. The year of date of diagnosis ranges from 1983 to 2015 (median: 2006). The median diagnosis year of patients with hearing loss is 2005 ranging from 1983 to 2015; while the median diagnosis year of non-hearing loss group is 2007 ranging from 1996 to 2013. There is no significant relationship between hearing loss and the year of diagnosis to the date of the last audiogram, [OR = 0.96 (0.90- 1.03), p- = 0.22)]. Subgroup analysis comparing 2010-2015 to 1990-1995 also shows the year of diagnosis has no significant relationship with hearing loss (p = 0.16).

Although we examined specific infusion parameters, we were unable to assess how patients may vary in CDDP exposure based on varying pharmacokinetics. Such variations could lead to variances in area underneath the curve (AUC) that could account for different levels of toxicity. Calvert established a formula to establish more consistent dosing and AUC attainment for carboplatin (26). Unfortunately, due to the unpredictable pharmacokinetics of cisplatin, comparable formulas have yet to be developed (18, 27). More research is needed in this field.

Despite these limitations and given the paucity of studies examining this subject, our audiologic monitoring program provided evidence that calls for new approaches for CDDP administration.

This project is one of the largest audiologic studies examining the influence of CDDP dosing and schedules on hearing loss. We demonstrated the amount of CDDP infused per dose is a significant risk factor. These findings support an observation in a previously reported clinical trial by the Children's Oncology Group where the two cohorts only varied with the amount of CDDP per dose with worse ototoxicity observed in the group assigned the higher dose (13). Such dosing can lead to higher peak serum levels that can lead to greater penetration into the cochlea. Given the recent studies demonstrating the persistent retention of CDDP in the cochlea, alternative dosing with lower amounts per dose may reduce CDDP accumulation in the cochlea and may potentially lead to less ototoxicity while retaining its anti-neoplastic properties. Prospective clinical trials are needed.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Washington University School of Medicine Human Research Protection Office. 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

Design of the trial – RH, SH, Execution of the trial – RH, SH, JH, KS, Data collection – SH, MC, AS, Data analysis – NW, Writing Manuscript – RH, SH, MC, AS, NW. All authors contributed to the article and approved the submitted version.

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A Prospective Study on Fertility Preservation in Prepubertal and Adolescent Girls Undergoing Hematological Stem Cell Transplantation

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Background: Hematological stem cell transplantation (HSCT) is an established method which has markedly increased the survival rate of hematologic malignancies since its introduction in the 1980's. The conditioning for HSCT has known gonadotoxic effects and often leads to premature loss of fertility. In this study we have prospectively followed a cohort of girls undergoing HSCT and studied the outcomes of fertility preservation treatments performed before or after HSCT, as well as the long-term reproductive outcome.

Methods: In this one-center prospective study, 39 girls counselled for fertility preservation prior to or after conditioning for HSCT for malignant or benign diseases at childhood or adolescence between 1990 and 2017 were included. The patients were presented with the option to undergo cryopreservation of ovarian tissue or oocytes depending on their age and the time available. Follicle counts of the ovarian tissue and number of oocytes collected before or after HSCT were compared between patients treated for benign and malignant diseases. Hormone measurements post HSCT treatment, including FSH and AMH, reproductive outcomes and overall survival until January 2021 were investigated.

Results: In total, 34 girls and adolescents underwent fertility preservation before or after HSCT. Before HSCT, ovarian tissue was cryopreserved in 15 patients and two patients had oocytes preserved. Thirteen patients cryopreserved ovarian tissue after HSCT and seven patients returned to cryopreserve oocytes. Follicles were present in all tissue samples collected prior to HSCT, and in more than half of the samples collected post-HSCT. Half of the patients had spontaneous menarche or resumed menstruation post HSCT. Overall, 35 patients had survived at end of follow up and 7 patients had achieved parenthood.

Conclusions: Since fertility loss is common following HSCT, fertility preservation should be offered to all patients. Fertility preservation treatments can be performed both before and after HSCT.

Clinical Trial Registration: https://clinicaltrials.gov/show/NCT04602962, identifier NTC04602962

Keywords: fertility preservation, cryopreservation, ovarian tissue, infertility, hematological stem cell transplantation, chemotherapy, gonadal toxicity, oocytes

INTRODUCTION

Survival of childhood cancer and severe anemias have improved over the past decades, in particular following the introduction of hematological stem cell transplantation (HSCT) in the 1980's.

Today HSCT is a well-established and often curative treatment option for severe benign and malignant diseases. HSCT in patients with leukemia is performed in remission, which means that before HSCT the girls have already been treated according to different leukemia protocols for months or years. The chemotherapeutic conditioning prior to HSCT, sometimes combined with total body irradiation, is known to seriously damage the gonads, which causes endocrine dysfunction, gonadal insufficiency and confers a high risk of permanent infertility in both sexes (1). Gonadal toxicity of HSCT has also been reported in children (2–4). In girls the ovarian toxicity of HSCT may result in early impairment or absence of pubertal development and premature ovarian failure (2, 5). The risk of premature ovarian failure seems to increase with increased age at HSCT and is higher if alkylating drugs or total body irradiation are used (1, 2, 6–8). In general, the conditioning required for HSCT in patients with a malignant diagnosis requires more intense and more gonadotoxic treatment compared to that of patients with benign diagnosis, where the administered treatment regime does not have the same crucial impact on patient survival. Late effects of HSCT have prompted the introduction of reduced impact consolidation protocols, which have shown a positive effect on post treatment fertility when implemented in a young population (9, 10).

Current programs for fertility preservation have been developed at many centers worldwide. If patients are promptly referred, the counselling and performance of fertility preservation does not delay the initiation of a planned cancer treatment (11–13). Since future fertility is one of the most important issues raised by adolescent patients and emerging adult cancer survivors (14), pediatricians, specialists in reproductive endocrinology, oncology, surgery and reproductive medicine as well as skilled laboratory resources are all increasingly involved in the complex process of providing services for fertility preservation to young patients and children. The decision of how much time is available to undergo fertility preservation before starting chemotherapy for treatment of cancer should be discussed with the treating oncologist. In some cases it is better to postpone ovarian tissue harvesting until after the patient has received chemotherapy and has a better health status. Although collecting reproductive cells or tissue after chemotherapy treatment is not optimal (5), the abundant ovarian reserve in young girls may allow for this strategy (15).

In Sweden and the other Nordic countries, programs for fertility preservation are offered free of charge at tertiary care university hospitals to all patients facing treatments with risk of subsequent infertility (16). For pre-pubertal girls the only available option for fertility preservation is ovarian tissue cryopreservation (17). Post-pubertal girls have the additional option to cryopreserve oocytes, provided there is time for controlled ovarian stimulation and the acceptance of transvaginal follicle aspiration to retrieve the oocytes (13).

The Fertility Preservation Program at the Reproductive Medicine Clinic of Karolinska University Hospital was initiated in the 1970's when methods for freezing sperm first became available. Since 1998 the program also included cryopreservation of embryos, ovarian tissue and thereafter oocytes, which may be elected by women, girls and transgender men before gonadotoxic treatments. All patients are currently followed as part of a long-term prospective observational study to evaluate the safety and efficacy of the treatments offered and results on this cohort have been reported (13, 18–21).

At present there are only a few studies reporting the long-term fertility and pregnancy outcome of girls undergoing fertility preservation before highly gonadotoxic chemotherapy due to severe benign or malignant disease (6, 13, 22, 23). In this study we report a prospective cohort of girls and adolescent women undergoing HSCT due to malignant and severe benign diseases, and the outcomes of fertility preservation treatments performed before or after HSCT, as well as the long-term reproductive outcomes and overall survival.

MATERIALS AND METHODS

Data Source and Study Population

This is a single center, prospective study on fertility preservation and long-term outcomes in young girls and adolescents who have undergone HSCT. The cohort was diagnosed with malignant or severe benign disease between 1990 and 2017 at ages 0-19, and referred to the Pediatric Oncology-Endocrinology and Reproductive Medicine Center (Karolinska University Hospital, Stockholm) either before or after their HSCT. Patients were followed for reproductive outcomes and mortality until January 31st 2021. All conditioning regimens for HSCT which included busulfan or melphalan, and regimens

with high doses of other alkylating chemotherapy in combination with total body irradiation, were categorized as having a high risk of causing infertility. Conditioning using lower doses of alkylating chemotherapy with or without total body irradiation were categorized as intermediate risk treatments.

Standardized Counselling of Girls and Teenagers

At the time of counselling, oral and written age adapted information on fertility preservation was provided by both a pediatrician and a specialist in reproductive medicine (13). The counselling of the patients and their families included information on available options for fertility preservation, as well as alternative ways of achieving future parenthood, such as egg donation or adoption. The possibility to return for fertility preservation after completed treatment was also offered.

Fertility Preservation Methods

Ovarian tissue retrieval was planned and performed within a few days, and scheduled 1-2-weeks before beginning a conditioning regimen and HSCT whenever possible. The surgery was performed laparoscopically under general anesthesia, and usually planned simultaneously with other necessary procedures such as a central line insertion. Ovarian biopsies or unilateral oophorectomy was performed based on ovarian size and the treatment protocol. The ovarian cortex was sliced into small pieces (5 x 5-10 mm, with a thickness of about 1 mm) and cryopreserved by slow freezing or vitrification. In order to cryopreserve oocytes, ovarian stimulation and oocyte retrieval were presented as an option to adolescents post menarche. Ovarian stimulation with gonadotropins requires 1-2 weeks of gonadotropin stimulation prior to trans-vaginal follicle aspiration. The procedures for oocyte pick-up were performed under sedation and local anesthesia.

Histopathology

Histopathological analysis of ovarian tissue was performed at the Department of Clinical Pathology and Cytology, Karolinska University hospital. One piece of the tissue was used to assess the presence of follicles and estimate the follicular density. Evaluation for presence of malignant cells was also requested.

Patient Follow-Up

After completion of chemotherapy and HSCT, young pre-pubertal girls were followed at the Pediatric Oncology and Pediatric Endocrine units. Pubertal progression and hormonal levels were evaluated. Measurements of follicle stimulating hormone (FSH) and anti-Müllerian hormone (AMH) were performed at the Central Laboratory for Clinical Chemistry, Karolinska University Hospital. The pediatric endocrinologist was responsible for the initiation of puberty induction and hormone replacement during adolescence.

Patients were encouraged to return to the reproductive clinic after completed chemotherapy to evaluate their remaining ovarian reserve. If no fertility preservation was performed at the time of HSCT treatment due to lack of referral, time restraint or by patient's choice, cryopreservation of ovarian tissue or oocytes was presented as an option post HSCT as long as some ovarian activity remained.

Statistical Analyses

Outcomes of fertility preservation treatments were compared between patients with benign and malignant diagnosis, and between patients before and after HSCT. Pearson chi-square test was used for categorical variables, and Wilcoxon rank-sum test for continuous variables due to non-normal distributions.

Data management and analyzes were performed using Stata (StataCorp. 2019. Stata Statistical Software: Release 16: StataCorp LLC). All tests were two sided with a significance level of 5%.

RESULTS

The cohort included 39 female patients, aged 0-19 years at time of diagnosis, referred for fertility counselling between October 1998 and June 2020 (**Table 1**). The indication for HSCT was a malignant disease in 25 patients and severe hematologic disease in 14 patients. The most common malignant diagnoses were leukemia, lymphoma and sarcoma, while aplastic anemia and thalassemia were the most common benign diagnoses. Before HSCT, 21 patients with malignant disease underwent conditioning with a high risk of causing infertility and 4 patients received intermediate risk treatment. HSCT was given to 17 patients in first remission and 8 patients in second remission. For benign diseases, the conditioning regimen was classified as high risk in 6 patients and intermediate risk in 8 patients. After counselling, 34 of the 39 referred patients underwent fertility preservation. The five patients who did not undergo FP had received high risk conditioning for HSCT due to malignant disease in ages 0-4 years. They were counselled 9-17 years after HSCT, at which time they had high FSH (>20 IU/L) and undetectable AMH (<0.05 μg/L; one missing).

Ovarian Tissue Cryopreservation

Ovarian tissue cryopreservation was performed in 28 patients (**Table 2**). Among 16 patients with malignant disease, eleven were biopsied before HSCT, five were in second remission. The remaining five patients with malignant indications had undergone HSCT with high risk conditioning at the time of the biopsy. Eight of the 12 patients with benign disease had undergone HSCT at the time of the biopsy, whereof four had received high risk conditioning. The median age at ovarian tissue cryopreservation was 15 years in patients with benign disease and 13 years in patients with malignant disease. Ovarian biopsies of varying size were performed in 24 patients and unilateral oophorectomy was performed in 4 patients.

Histopathological analysis of one piece of the ovarian tissue was performed in 21 samples. Of these samples, 11 were taken prior to HSCT and 10 post HSCT (**Table 3**). Follicles were found in 17 of the 21 samples examined. Follicles were found in all 11 analyzed samples taken prior to HSCT, and in 6 of 10 samples

TABLE 1 | Description of cohort.

Diagnosis	Benign disease n = 14		Malignant disease n = 25	
	No.	%	No.	%
Acute lymphocytic leukemia (ALL)			5	20.0
Acute myelogenous leukemia (AML)/Myelodysplastic syndrome (MDS)			7	28.0
Other leukemia			5	20.0
Lymphoma			4	16.0
Sarcoma			2	8.0
Neuroblastoma			2	8.0
Aplastic anemia	5	35.7		
Thalassemia major	3	21.4		
Amegakaryocytic thrombocytopenia	2	14.3		
Other hematological disease	3	21.4		
Other benign disease	1	7.1		
Age at diagnosis				
0-4	8	57.1	9	36.0
5-9	5	35.7	3	12.0
10-14	1	7.1	8	32.0
15-19	0	0.0	5	20.0
Age at HSCT				
0-4	4	28.6	7	28.0
5-9	4	28.6	5	20.0
10-14	4	28.6	6	24.0
15-22	2	14.3	7	28.0
Year of HSCT				
1990-1999	6	42.9	2	8.0
2000-2009	7	50.0	17	68.0
2010-2018	1	7.1	6	24.0
Conditioning for HSCT				
High risk of causing infertility	6	42.9	21	84.0
Intermediate risk of causing infertility	8	57.1	4	16.0
First fertility preservation treatment				
Before HSCT	4	28.6	13	52.0
After HSCT	10	71.4	7	28.0
Not yet performed	0	0.0	5	20.0

HSCT, Hematological stem cell transplantation.

taken after HSCT (p=0.020). The density of follicles varied from 1 to 1519/mm^2 in the samples containing follicles. There was no significant difference in median follicle density between samples from patients with benign and malignant disease (median 25 vs 49, p=0.721), or between samples taken before and after HSCT (median 56 vs 23, p=0.268). While the sample with the highest follicle density (1519/mm^2) was taken after HSCT with high risk conditioning from a patient who was in second remission, the general trend was that samples taken after HSCT were less likely to contain any follicles (100% vs 60%, p=0.020). Among the patients with high risk conditioning for malignant disease who preserved ovarian tissue after HSCT, follicles were found in two of five analyzed samples. Among the eight patients with benign disease who cryopreserved ovarian tissue after HSCT follicles were found in four of five samples analyzed, three from patients who had undergone high risk conditioning and one after intermediate risk conditioning.

Oocyte Cryopreservation

Two patients with malignant disease, and none with benign disease, received ovarian stimulation for oocyte cryopreservation

TABLE 2 | Ovarian tissue cryopreservation by indication.

	Benign, n=12	Malignant, n=16	p-value*
Age, median (range)	15 (10-19)	13 (8-21)	0.242
Follicles present, n (%)			
Yes	8 (89%)	9 (75%)	0.422
No	1 (11%)	3 (25%)	
Follicle density per mm^2, median (range)	25 (0-527)	49 (0-1519)	0.721

**Calculated using Chi-square test for proportion of samples containing follicles and Rank-sum test for age and follicle density. HSCT, Hematological stem cell transplantation.*

TABLE 3 | Ovarian tissue cryopreservation before and after HSCT.

	Before HSCT, n=15	After HSCT, n=13	p-value*
Age, median (range)	13 (8-21)	14 (11-17)	0.471
Follicles present, n (%)			
Yes	11 (100%)	6 (60%)	0.020
No	0 (0%)	4 (40%)	
Follicle density per mm^2, median (range)	56 (1-1384)	23 (0-1519)	0.268

**Calculated using Chi-square test for proportion of samples containing follicles and Rank-sum test for age and follicle density. HSCT, Hematological stem cell transplantation.*

before HSCT. After HSCT, seven patients have received ovarian stimulation for oocyte cryopreservation (**Table 4**). Three of these patients had benign disease, median FSH 5.4 (range 1.0-5.8), and four had malignant disease, median FSH 6.8 (range 2.2-13.0). Two of the patients with malignant disease had received high risk conditioning in second remission, two had intermediate risk treatment in first and second remission, respectively, while all three with benign disease had intermediate risk treatment. Three of these seven patients had previously cryopreserved ovarian tissue. All patients cryopreserved at least one oocyte (median 5, range 1-13) after one or two stimulation cycles. The median age at first oocyte cryopreservation was 20 years in patients with benign disease and 16 years in patients with malignant disease.

Long Term Follow-Up

The cohort has been followed a median of 17 years after HSCT (range 0-31 years). Four girls who had leukemia died of their disease; 0, 0, 4 and 9 years after HSCT. All of these girls had cryopreserved ovarian tissue and none had cryopreserved oocytes.

After HSCT, 11 of 14 patients (86%) treated for a benign disease and 6 of 21 patients (29%) with malignant diseases had spontaneous menarche or continued having menstrual periods (p=0.001). Premature ovarian failure post HSCT has occurred in 3 of 14 patients (21%) with benign disease and 15 of 21 patients (71%) with malignant disease (p=0.004). One patient resumed her periods but later during follow up experienced premature ovarian failure and data are missing for 4 patients.

At the end of follow-up (January 31st 2021), 28 of the 34 patients who had cryopreserved ovarian tissue or oocytes were alive and at least 20 years of age (median 28, range 20-39). So far, seven patients have at least one child. The 21 adult women who did not yet have children at the end of follow-up were younger than the 7 women who had at least one child (median age 27 vs 31, p<0.001). None of the women that conceived had stored oocytes. None of the nine women with cryopreserved oocytes has yet returned for utilization.

Among the women with previous benign diagnoses, three conceived naturally and one using sperm insemination. Two of the women that conceived naturally had received high risk conditioning. Three patients of this cohort have re-transplanted ovarian tissue, all of whom had received high risk conditioning. All proceeded with ovarian stimulation, where two led to successful oocyte pickup and embryo transfer, and one woman treated with HSCT for a malignant disease in first remission has conceived. Additionally, two women treated for malignant diseases have achieved parenthood, one through oocyte donation and one has adopted a child. Both these women received high risk conditioning for HSCT in second remission.

FSH post HSCT was evaluated in 33 patients and the levels were significantly higher in the group treated for malignant disease than in the group treated for benign diseases (median 29.5 mIU/mL, range 2.2-127, and median 5.6 mIU/mL, range 1-58, respectively, p=0.016). AMH was measured in 23 patients post HSCT and had a median value of 0.5 μg/L (range 0-1.9) in the group treated for benign diseases and 0.08 μg/L (range 0-1.3) in the group treated for malignancy (p=0.235).

TABLE 4 | Oocyte cryopreservation after HSCT, by indication.

	Benign, n = 3	Malignant, n = 4	p-value*
Age, median (range)	20 (17-26)	16 (14-20)	0.400
Time since HSCT, median (range)	11 (8-19)	8 (5-10)	0.229
AMH, μg/L, median (range)	0.62 (0.20-0.63)	0.85 (0.30-1.30)	0.629
FSH, IU/L, median (range)	5.4 (1.0-5.8)	6.8 (2.2-13.0)	0.229
Number of oocytes, first stimulation, median (range)	4 (3-7)	1 (0-12)	0.343

**Calculated using Rank-sum test. HSCT, Hematological stem cell transplantation.*

DISCUSSION

Our study on fertility preservation including the use of experimental methods such as cryopreservation of gonadal tissue for pre-pubertal and adolescent children of both sexes has been ongoing since 2002 (18). In this cohort 39 girls and teenagers undergoing HSCT for treatment of malignant or severe benign diseases received counselling on fertility preservation and 34 patients choose to proceed whereas five chose not to proceed with fertility preservation after counselling. The high level of participation among the included patients reflects the need for fertility counselling even at a young age. This is supported by studies showing that fertility is among the main concerns for young patients (24, 25). A total of 28 patients in the cohort cryopreserved ovarian tissue and nine could cryopreserve oocytes. Fertility preservation procedures could be performed before HSCT in 17 patients. The rate of fertility preservation after HSCT was 51% (20/39), this includes both patients who had not undergone previous fertility preservation procedures and patients who had previously cryopreserved ovarian tissue. An additional attempt at fertility preservation through oocyte cryopreservation can be of extra importance for patients with hematological malignancies where re-transplantation of tissue might reintroduce the malignancy.

Our results indicate that HSCT negatively impacts fertility in all patients, and more noticeably so in the patient group with malignant disease. Due to the small number of patients in our cohort who received total body irradiation, we were not able to assess the negative impact on fertility shown in previous studies (1, 2, 6–8). In the cohort of girls with a malignant disease we observed a significantly higher risk for premature ovarian failure and a lower chance of finding follicles in ovarian tissue retrieved after HSCT treatment. Adolescents who had resumed their menses could successfully undergo oocyte cryopreservation after HSCT. Among these patients, median AMH, number of oocytes retrieved and FSH was not significantly different between patients with malignant and benign disease, nor was FSH above the normal value for women in fertile age, which is in accordance with previous studies (26). However, FSH measured in all patients show the gonadotoxic effect of the treatments, visible especially among the patient group treated for malignant disease.

It is also worth noting that, when looking at the full patient group with malignant disease, the measured AMH median lies well below the average for the age group, and median FSH well above (26, 27). These results are in line with the previously observed trend among women who returned after fertility preservation to attempt pregnancy, where a significantly lower live birth rate has been found among survivors of malignant disease when compared to women with a previous benign indication (6, 13, 22, 23).

The results suggests that referral to fertility counselling and treatment before HSCT is of outmost importance for patients undergoing HSCT. While oocyte cryopreservation might still be the preferred option for fertility preservation, cryopreservation of ovarian tissue is quickly becoming an established option for successful pregnancy and should be encouraged in young women and cases with time limitations (28). There have also been attempts to culture follicles *in vitro* to obtain mature oocytes from ovarian tissue with promising results (29).

This study is limited by the small size and the heterogeneity of the cohort. Although the study is prospective with long-term follow-up of the patients, information on fertility treatment attempts or live births occurring outside our center may have been missed. The age of the women with successful pregnancies is significantly higher than the mean age of the total cohort, which is lower than the mean age for first time mothers in Stockholm (30). In addition, considering the demanding treatments that the patients in the cohort have undergone, it is likely that the utilization rate will increase with longer follow-up. To better predict fertility after HSCT, additional factors such as the combined effects of age, treatment regime and individual trends in the oocyte reserve need to be explored in larger cohort studies.

CONCLUSIONS

The results of this study underscore the need for fertility preservation in prepubertal and adolescent girls planned for HSCT treatment due to the gonadal toxicity inherent to the HSCT conditioning. Timely fertility counselling and the option of fertility preservation should be offered to all young female patients prior to or even after HSCT, whenever possible.

Today we lack tools to accurately predict which patients will lose their fertility, although patients with malignant diagnoses are at a greater risk compared to patients treated for benign diagnoses. Ovulation and fertility can be retained after HSCT but premature ovarian insufficiency early in life, before the patient plans to start a family, is a considerable risk. Our study shows that fertility preservation can be achieved before and also after HSCT and that these procedures enhance the chances of future fertility. Patients who have previously cryopreserved ovarian tissue may benefit from additional oocyte cryopreservation, as it reduces the chances of reintroducing malignancy through the transplant and the use of vitrified oocytes is now established at most reproductive centers. Fertility counselling and evaluation of the remaining fertility potential even after HSCT treatment in childhood can also provide an opportunity to undergo fertility preservation during adolescent years.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical approval for the study and for the follow-up of patients to adulthood was granted by the Ethical Review Board of Karolinska University Hospital (Dnr 427/03) and the Regional Ethics Committee of Stockholm (Dnr 2011/1158-31/2, 2014/470-32, 2016/2530-32 and 2018/2255-32). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KR-W designed the study and provided economic and administrative support. IW and KR-W collected the data. IW and FL analysed the data. IW, FL, HN, BB, and KR-W drafted and wrote the manuscript and revised the content. All authors contributed to the article and approved the submitted version.

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Follicle Rescue From Prepubertal Ovaries After Recent Treatment With Cyclophosphamide—An Experimental Culture System Using Mice to Achieve Mature Oocytes for Fertility Preservation

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Ovarian tissue cryopreservation is the only feasible method for fertility preservation in prepubertal girls that will undergo gonadotoxic chemotherapy. To date, the only clinical use of cryopreserved tissue is by a later tissue retransplantation to the patient. Clinical challenges in fertility preservation of very young patients with cancer include time constraints that do not allow to retrieve the tissue for cryopreservation before starting chemotherapy and the preclusion of future ovarian tissue transplantation due to the risk of reintroduction of malignant cells in patients with systemic diseases. To overcome these two challenges, we investigated using an experimental model the feasibility of retrieving secondary follicles from ovaries of prepubertal mice after cyclophosphamide (CPA) treatment in increasing doses of 50, 75, and 100 mg/kg. The follicles were thereafter cultured and matured *in vitro*. The main outcomes included the efficiency of the method in terms of obtained matured oocytes and the safety of these potentially fertility preservative procedures in terms of analyses of oocyte competence regarding normality of the spindle and chromosome configurations. Our findings demonstrated that it was feasible to isolate and culture secondary follicles and to obtain mature oocytes from prepubertal mice ovaries recently treated with CPA. The efficiency of this method was highly demonstrated in the 100 mg/kg CPA group, with near 90% follicle survival rate after 12 days' culture, similarly to control. Around 80% of the follicles met the criteria to put into maturation, and more than 40% of them achieved metaphase II, with normal spindle and chromosome configurations observed. Suboptimal results were obtained in the 50 and 75 mg/kg CPA

groups. These paradoxical findings towards CPA dose might probably reflect a more difficult selection of damaged growing follicles from ovaries recently treated with lower doses of CPA and a hampered ability to identify and discard those with reduced viability for the culture.

Keywords: female fertility preservation, cyclophosphamide, *in vitro* culture and maturation, prepubertal ovary, chemotherapy, ovarian follicle isolation

INTRODUCTION

Improvements in diagnostic methods allowing early cancer diagnosis and improvements in cancer treatment have both increased cancer patients' survival rate globally. However, cancer treatment, especially chemotherapy and radiotherapy, can cause premature ovarian failure and infertility (1). For a young cancer survivor, this is a hard pathway to face. Thus, efforts have been made to develop methods for fertility preservation, and current guidelines recommend timely discussions with young adult patients, children, and their families on feasible fertility preservative methods as early as possible before the treatment starts aiming at offering the full range of options (2, 3).

Although well-established female fertility preservative methods including the cryopreservation of mature oocytes or embryos are available worldwide for adult patients, these are not applicable to young girls. Ovarian tissue cryopreservation can be offered instead in these cases (4). The method does not require hormonal stimulation, and the tissue is usually retrieved using minimally invasive surgery, which has been reported by programs for fertility preservation (5). However, many young patients need to start a treatment without delay, and data are lacking regarding the usefulness of ovarian tissue retrieved after the chemotherapy rounds have been already initiated.

Up to date, the only currently developed method to regain fertility using the cryopreserved ovarian tissue is by retransplantation of the tissue to the patient. The ovarian follicles will then grow and develop to allow the performance of assisted reproductive treatments or even natural conceptions (6). A few hundreds of successful cases using cryopreserved ovarian tissue have been reported worldwide; however, a high number of women cannot undergo transplantation due to the risk of reseeding malignant cells back within the transplanted tissue. There is hence an urgent need for developing novel methods to use the ovarian tissue in the future, as the numbers of women undergoing these procedures are increasing (7).

In this study, we approached two challenging clinical situations using a translational research model. We wished to investigate the feasibility of follicle isolation from prepubertal mice ovaries recently treated with a gonadotoxic cytostatic drug, cyclophosphamide (CPA) (8), and the reproductive potential of these follicles regarding the final achievement of mature competent oocytes. Three different doses of CPA were tested *vs*. a control group without treatment. In all groups, isolated follicles were thereafter *in vitro* cultured and matured. The culture system allowed the evaluation of individual follicle growth, hormonal production, oocyte maturation, and spindle structure and chromosomal configurations in mature oocytes. To our knowledge, there is limited knowledge on the feasibility of performing fertility preservative procedures after gonadotoxic cancer treatment has been initiated, especially at prepubertal stage (9, 10).

Abbreviations: CPA, cyclophosphamide; α-MEM, α-minimal essential medium; MII, metaphase II; AMH, anti-Müllerian hormone; IVM, *in vitro* maturation; PFD, primordial follicle depletion; HSCT, hematologic stem cells transplantation.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma-Aldrich® or Gibco, Thermo Fisher Scientific®, unless otherwise indicated.

Animals and Grouping

Twenty in-house breeding 12-day-old B6CBA/F1 female mice were randomly assigned (n = 5/group) into three groups treated with different doses of CPA (100, 75, or 50 mg/kg) or into a control group without treatment. CPA was freshly prepared in a 0.9% NaCl solution and intraperitoneally injected. All the mice were sacrificed 3 days after CPA injection, and the ovaries were collected into Leibovitz's 15 media supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, at 37°C. The doses of CPA chosen for this study have been validated in previous experimental studies that have also considered their equivalence to human treatment (11, 12).

All animal procedures were approved by Karolinska Institutet and the regional ethics committee for animal research in accordance with the Animal Protection Law, the Animal Protection Regulation, the Regulation of the Swedish National Board for Laboratory Animals, identified Dnr 1372 (date: January 24, 2018). All procedures were conducted in accordance with accepted standards of humane animal care.

Follicle Isolation, *In Vitro* Culture, and Maturation

Follicle isolation was performed immediately after ovary collection. Under a stereomicroscope (Nikon®), the ovaries were cleaned up of the surrounding tissue, and follicles were isolated mechanically using micro-fine U-100 insulin syringes (0.3 ml, BD Medical). Secondary follicles with 100–130 μm diameter, two or more layers of granulosa cells, a round and central oocyte, and some theca cells attached were selected for culture. Ovarian follicle culture was performed as previously

described (10, 13, 14). Briefly, selected secondary follicles were individually cultured in drops of a droplet system (20 µl per drop, 10 drops per dish) containing culture medium. The culture medium was α-minimal essential medium (α-MEM) GlutaMAX supplemented with 5% fetal bovine serum, 10 µg/ml transferrin, 5 µg/ml insulin, and 100 mIU/ml recombinant follicle-stimulating hormone (GONAL-F). The droplet system was covered with mineral oil and kept in a humidified incubator at 37°C with 5% CO_2. Isolation, selection, and start of culture day was designated as day 0 and the last day as day 12. Every other day, the follicles were observed under an inverted microscope (100×) (Nikon®) to record morphological characteristics and follicular size using a calibrated ocular micrometer (Nikon®). Follicular size was estimated as the mean diameter obtained with two perpendicular measures including the granulosa cell mass without the theca cells. Culture medium was renewed every other day by collecting 10 µl of culture medium and adding 10 µl of fresh culture medium. On culture days 4, 8, and 12, the culture medium collected was diluted in 90 µl α-MEM GlutaMAX with bovine serum albumin (40 mg/ml) and stored at −20°C for further analysis.

On day 12 of culture, follicles that reached at least 200 µm of diameter, presented a clear granulosa cell proliferation, and had a visible round oocyte were classified as growing follicles that survived throughout the *in vitro* culture. Additionally, follicles with at least 450 µm diameter were selected for *in vitro* maturation. *In vitro* maturation was performed by transferring the selected follicles at day 12 to a microdrop system similar to the culture system, but in which 1.5 IU/ml recombinant human chorionic gonadotrophins and 5 ng/ml recombinant epidermal growth factor were added to the culture medium. Follicles were incubated in a humidified atmosphere at 37°C and 5% CO_2, and maturation status was verified 16–20 h later. After verifying the cumulus–oocyte complex formation, the oocytes of the follicles that formed cumulus–oocyte complex were denuded, and their maturation status were evaluated under an inverted microscope. Oocytes with a visible polar body were classified as mature oocytes (metaphase II, MII).

Hormone Assays

The culture medium collected on days 4, 8, and 12 were used to measure the secretion of 17β-estradiol and anti-Müllerian hormone (AMH). Hormonal assays were performed using commercially available enzyme-linked immunoassay kits for 17β-estradiol (ab108667, Abcam) and AMH (RK02588, ABclonal) following the manufacturers' protocols. The limits of sensitivity for 17β-estradiol and AMH were 8.68 and 53.3 pg/ml, respectively. For each hormone, duplicate measurements were performed using the collected culture medium at days 4, 8, and 12 of culture. For each time, in each group, the culture media of five follicles with similar growth features and from which resulted mature oocytes were pooled together to reach the required volume sample amount for the assay.

Spindle and Chromosome Analysis of *In Vitro* Matured Oocytes

After *in vitro* maturation (IVM), denuded mature oocytes (MII) were selected and washed in a washing buffer (Dulbecco's phosphate-buffered saline) containing 0.1% polyvinyl alcohol. The oocytes were then fixed with 2% formaldehyde in washing buffer containing 0.2% Triton X-100 for 40 min. After fixation, the oocytes were incubated overnight at 4°C in a blocking buffer (washing buffer supplemented with 1% bovine serum albumin). Then, the oocytes were incubated for 40 min in the blocking buffer supplemented with 10% fetal bovine serum. Antibodies used were diluted in the blocking buffer. The oocytes were incubated with mouse monoclonal anti-α-tubulin antibody (T9026, 1:1,000) for 45 min followed by a 40-min incubation with Alexa Fluor 488-labeled goat antimouse IgG antibodies (ab150113, Abcam, 1:200) at 37°C. Then, 10 µg/ml propidium iodide (81845) was added to the oocytes for 20-min incubation. Finally, oocytes were mounted between a coverslip and a microscope slide with Prolong Diamond Antifade mountant (P36965, Invitrogen). All the above-described procedures were performed under a stereomicroscope. The slides were kept at 4°C until confocal imaging.

Labeled tubulin and chromatin were assessed using a Nikon Eclipse Ti microscope equipped with the appropriate filter sets for analyzing Alexa Fluor 488 and propidium iodide with 100× oil immersion objective. Four to six MII oocyte images in each group were captured with an Andor iXon Ultra camera and analyzed using NIS Elements program. Image analyses were performed using Fiji software.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 8.4.3 software package. Differences in follicular sizes, hormones levels on different culture days, and follicle yield per ovary between groups were tested by multiple t-tests. Comparisons of parameters other than follicle yield in **Table 1** between CPA treated groups and control were performed by chi-square test (two-sided).

TABLE 1 | Follicle yields per ovary, *in vitro* growth characteristics, and oocyte maturation status achieved by CPA-treated groups in comparison with controls.

	Ovaries N	Cultured follicles N	Follicle yields/ovary Mean ± SD	Attached within first 2 days N (% = 100%*N/Cultured follicles)	Survival (Day 12) N (% = 100%*N/Cultured follicles)	Put into maturation N (% = 100%*N/Cultured follicles)	MII oocytes N (% = 100%*N/Cultured follicles)
Control	9	134	14.9 ± 4.4	113 (84.3%)	119 (88.8%)	107 (79.8%)	69 (51.5%)
50 mg/kg	9	170	18.9 ± 6.4	145 (85.3%)	133 (78.2%)[a]	115 (67.6%)[a]	56 (32.9%)[b]
75 mg/kg	9	211	23.6 ± 5.5[b]	178 (84.4%)	172 (81.5%)	148 (70.1%)[a]	61 (28.9%)[c]
100 mg/kg	9	144	16.0 ± 5.1	130 (90.3%)	129 (89.6%)	110 (76.4%)	60 (41.7%)

[a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$.

RESULTS

Secondary Follicle Isolation Yield, *In Vitro* Growth, and Maturation

A total of nine ovaries per group were used in this study. The yield of secondary follicles isolated per ovary did not differ significantly between 50 or 100 mg/kg CPA groups *vs*. control; however, in the 75 mg/kg group, the yield was significantly higher *vs*. control ($p < 0.01$, **Table 1**).

The *in vitro* growth behavior of follicles during culture was similar among all groups regarding percentages of follicles attached to the culture dish during the first 2 days (**Table 1**) and growth curve features (**Figure 1**). Overall, the mean follicular size steadily increased throughout the culture but with different paces at different culture periods, with slower growth during the first half of culture and faster during the second half (**Figure 1**). The initial mean follicle diameter was 116.2 μm among all the groups (**Table 2**). By the end of culture, follicles in all groups reached mean sizes ranging from 631.8 to 655.9 μm (**Table 2**) without significant differences. After 12 days in culture, nearly 90% of the follicles from the control and the 100 mg/kg groups survived. However, a lower follicle survival was observed in the 50 mg/kg CPA group (78.2%) *vs*. control (88.8%), $p < 0.05$ (**Table 1**). By the end of culture, significantly lower percentages of follicles reached the criteria to put into maturation in the 50 or 75 mg/kg CPA groups *vs*. control, whereas similar percentages were observed in the 100 mg/kg and control groups.

After IVM, more mature oocytes were obtained in the control group (51.5%) compared with the 50 and 75 mg/kg groups, where 32.9% and 28.9% were matured, respectively (**Table 1**). In the group treated with 100 mg/kg CPA, a similar percentage of mature oocytes was obtained compared to the control.

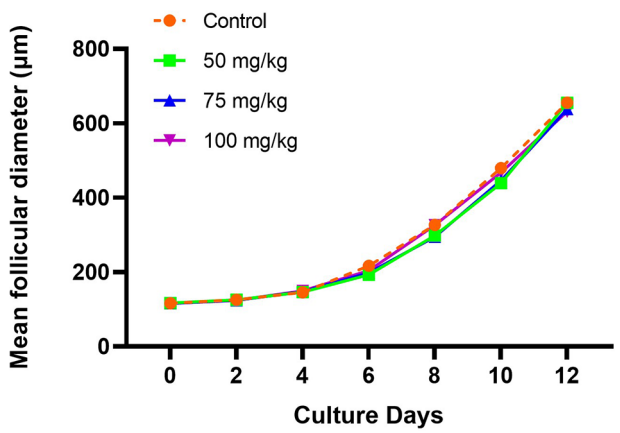

FIGURE 1 | Follicular growth curve in each group during the culture (X, culture days; Y, mean follicular diameter, μm).

Hormone Assays

Follicle secretion of 17β-estradiol determined in culture media was similar among all groups, increasing from day 4 to 12 (**Figure 2A**). On the last day of culture (day 12), the levels of 17β-estradiol detected were similar among controls and the 75 and 100 mg/kg CPA groups. However, a lower secretion of 17β-estradiol was detected in the 50 mg/kg CPA group, compared to control (11,023.57 pg/ml *vs*. 12,087.9 pg/ml, $p < 0.005$).

Initial AMH secretion measured in culture medium on day 4 showed similar levels between control and 50 mg/kg CPA groups, whereas a significantly higher initial AMH secretion was found in the 75 and 100 mg/kg CPA groups *vs*. control (**Figure 2B**). Over time, the AMH secretion gradually declined in the 50 and 75 mg/kg CPA groups and control, and the levels at day 12 did not differ among those groups *vs* control. However, the 100 mg/kg CPA group maintained a steady pace throughout the culture, and on day 12, the AMH level was 3.7 times of control ($p < 0.0001$).

Spindle and Chromosome Configurations in Mature Oocytes

In a normal MII oocyte, the spindle is bipolar barrel shaped, and the chromosomes are well-aligned on the metaphase equator in the center of the spindle. Within the MII oocytes observed, there was a trend that the spindle and chromosome structures in 100 mg/kg CPA group were more similar to control, whereas spindle and chromosome abnormalities were frequently observed in 75 and 50 mg/kg CPA groups. As shown in **Figure 3**, mature oocytes in the control group displayed organized chromosomes at the center of the bipolar barrel-shaped spindle. Whereas in the 75 and 50 mg/kg CPA groups, chromosome misalignment and spindle defects were observed in mature oocytes. Chromosomes were, even though at the center of the spindle, not well-aligned on the metaphase equator; meanwhile fragmentary spindles were also observed. In mature oocytes obtained from the 100 mg/kg CPA group, the spindle was bipolar barrel shaped, and the chromosomes were well-aligned on the metaphase equator.

DISCUSSION

This study was designed to investigate the feasibility of obtaining mature oocytes after *in vitro* culture of follicles isolated from prepubertal mice recently treated with CPA. The follicle culture

TABLE 2 | Dynamic follicular sizes in each group during the culture (mean follicle diameter ± standard deviation, μm).

	D0	D2	D4	D6	D8	D10	D12
Control	116.0 ± 9.3	125.5 ± 14.6	145.8 ± 27.3	217.3 ± 102.8	326.8 ± 166.8	479.6 ± 191.9	655.9 ± 190.0
50 mg/kg	116.9 ± 8.6	125.5 ± 10.8	146.6 ± 27.3	192.7 ± 83.9	298.1 ± 157.6	439.1 ± 209.6	655.0 ± 199.3
75 mg/kg	116.4 ± 8.3	124.4 ± 12.4	147.5 ± 27.1	198.4 ± 73.1	294.7 ± 144.6	446.2 ± 187.0	638.0 ± 193.5
100 mg/kg	115.6 ± 8.5	124.5 ± 12.8	149.5 ± 32.0	204.3 ± 92.5	326.3 ± 169.9	467.6 ± 192.2	631.8 ± 179.3

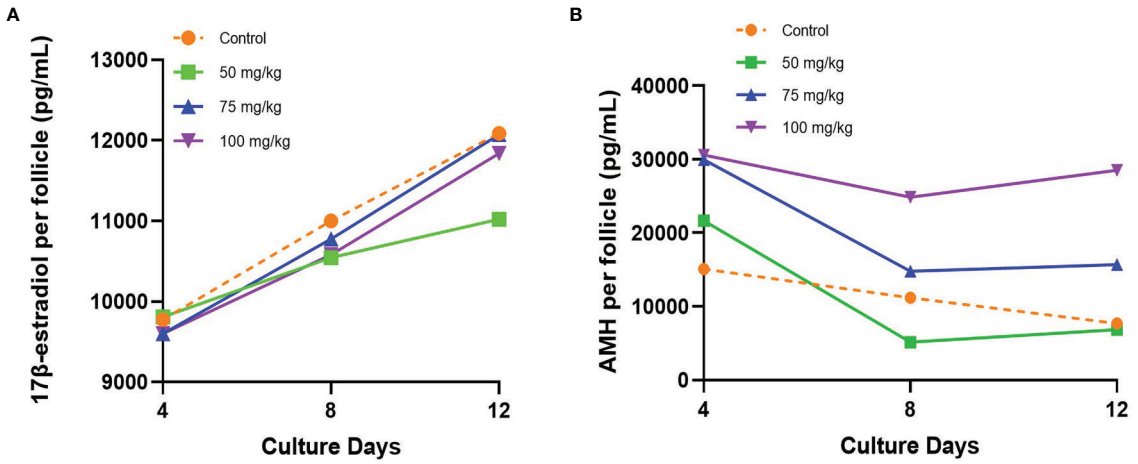

FIGURE 2 | **(A)** 17β-estradiol and **(B)** AMH secreted by individual follicle into the culture medium during the culture.

and oocyte maturation methods have been previously validated in mice, and pups have been obtained using those methods (13–15). Spindle and chromosome arrangements were analyzed in this study to evaluate the quality of the mature oocytes obtained.

The ovary is a complex organ, where multiple regulations among activated follicles determine the ultimate fate of each individual follicle, whether it will proceed towards further growth or become atretic, resulting in the elimination of more than 90% of the activated follicles (16). Meanwhile, there is a critical surveillance system in the ovary, to remove dead follicles and follicles with damage (17–19). In our study, ovaries post CPA treatment may have had a proportion of secondary follicles physiologically impaired, and some were probably already targeted by the surveillance system due to CPA-induced damage. In our experimental conditions, we removed the secondary follicles from the ovarian environment to culture them individually, allowing them to escape the physiological inhibition and the surveillance system with the final aim to grow till mature oocytes.

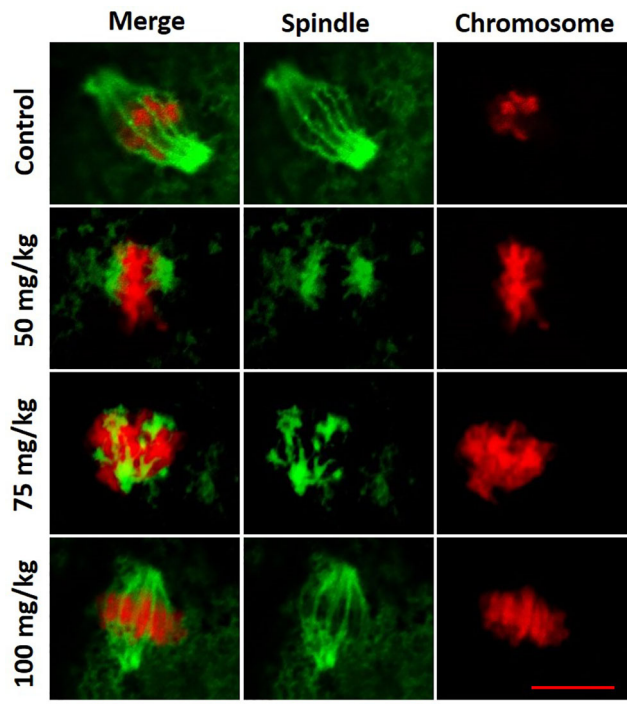

FIGURE 3 | Representative figures of spindle and chromosome configurations in MII oocytes from each group. Spindle fibers were detected by immunofluorescence for α-tubulin (green), while DNA was stained with propidium iodide (red). Scale bar, 10 μm.

Our findings showed that a large proportion of secondary follicles isolated from the ovaries of prepubertal mice after recent CPA treatment were not seriously affected by the CPA treatment. Those follicles could be supported to grow, and had spindle and chromosome configurations that were normal in the mature oocytes obtained after IVM, supporting the feasibility of our proposed method. Meanwhile, we found an interesting phenomenon towards the relationship between the efficiency of this method and the doses of CPA used, and we speculate this is somehow related to the mechanisms of how CPA induces primordial follicle depletion (PFD) in the ovaries.

According to the follicle selection criteria used in this culture system (13, 14), the yield of follicles/ovary obtained in the 75 mg/kg group was significantly higher and in the 50 mg/kg group slightly higher than that of control, whereas follicle yield was smaller in the 100 mg/kg group. This could support an early overactivation mechanism, which has been proposed to explain the final PFD induced by gonadotoxic chemotherapy (20–23), or it could indicate that in the two lower-dose groups of this study, the CPA dose was not high enough to immediately damage the follicles or trigger apoptosis in them. In 50 and 75 mg/kg groups increased activation might have allowed to pick more secondary follicles for culture; however, at 100 mg/kg, the follicle yield could have been additionally affected because the activated

follicles were more rapidly damaged due to a high CPA dose, and many of them did not met the criteria to be cultured.

The gonadal toxicity of CPA is dose dependent, as previously demonstrated (24, 25). In our study, the observed similar culture outcomes between 100 mg/kg CPA and the control groups, while poorer outcomes in 50 and 75 mg/kg CPA groups, were surprising. These might indicate that the initial selection of potentially viable secondary follicles was easier in the 100 mg/kg group, due to more serious recent CPA treatment induced damage causing morphologically recognizable changes. The visible changes allowed to discard follicles with damage and to select follicles that had survived the damage and probably were qualified to overcome the surveillance system, while this selection was more difficult in the 50 and 75 mg/kg groups. Thus, a higher proportion of secondary follicles with minor but not morphologically detectable damage was selected for culture in the 50 and 75 mg/kg groups. However, during culture, it was evident that the competency of these follicles was impaired, and a lower survival rate was observed after 12 days of *in vitro* culture. Additionally, lower percentages of cultured follicles from these groups reached the criteria for IVM, lower percentages of MII oocytes were obtained, and abnormal spindle and damaged chromosome structures were more frequently observed in the oocytes of these two groups.

The observed higher levels of AMH in 75 and 100 mg/kg CPA groups on day 4, and continuously higher in 100 mg/kg CPA group till day 12, might also support plausible primordial follicle activation after CPA *in vivo* treatment (20–23). This is not contradictory to the observation of reduced serum AMH levels by CPA treatment in studies *in vivo* (26, 27). Overactivation *in vivo* can initially lead to follicles sense through molecular communications and induce enhanced secretion of AMH to inhibit further overactivation through a paracrine regulation (28–30). Thereafter, the activated follicles may suffer direct damage by CPA or by lacking of growth support; thus, the AMH secretion becomes reduced in the long run. In this study, 72 h after CPA treatment, the activated follicles were isolated and individually cultured *in vitro*, leaving them free from subsequent CPA damage, but the initial effect that enhanced AMH production seemed to be kept, and the length of duration keeping this feature seemed to be dose dependent. As an alternative explanation, AMH could promote the growth of preantral follicles during *in vitro* culture through an autocrine effect, as supported by some studies (31–33). In our study, follicles isolated from 100 mg/kg CPA-treated mice ovaries might had been real survivors, secreting more AMH to promote their own growths. More investigations will be needed to further explain this phenomenon.

Since it is known that CPA is genotoxic (34), thus it is important to investigate the normality of the mature oocytes obtained after recent CPA treatment to guarantee the safety of our method. In addition to spindle and chromosome structure analysis, more investigations should be performed in the future, such as chromosome aberration tests and the final fertilization ability of the oocytes to evaluate normality of embryo development. On the other hand, clinical data from young female patients that have undergone ovarian tissue cryopreservation after several chemotherapy rounds and even after hematologic stem cells transplantation (HSCT) has demonstrated presence of primordial ovarian follicles in the cryopreserved tissue when the ovarian tissue was retrieved during childhood or adolescence (35). Moreover, ovarian tissue retrieved following HSCT conditioning at pubertal age has demonstrated full functionality after retransplantation, allowing two normal pregnancies, as recently reported (36).

Our experimental model deserves further translational investigation using human ovarian tissue. Methods for isolation and culture of human ovarian follicles obtaining mature oocytes have been reported (37). Further development of the methods hereby described is needed for the establishment of fertility preservative methods that can be performed after initiation of gonadotoxic chemotherapeutic treatment and that overcome the need of ovarian tissue retransplantation.

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 animal study was reviewed and approved by Karolinska Institutet and the regional ethics committee for animal research.

AUTHOR CONTRIBUTIONS

XH, AA, and KR-W designed the experimental setting for the study. XH, AA, LV-R, ASL, CD and SAdM performed the experiments. XH had the main responsibility for data analysis and wrote the first manuscript draft. All authors critically revised the manuscript. K-RW provided administrative support and funding. All authors contributed to the article and approved the submitted version.

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